UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE FARMACIA

TESIS DOCTORAL

Cárnicos funcionales enriquecidos en extracto de algarroba (Ceratonia siliqua, L.) en la prevención y tratamiento de la Diabetes Mellitus tipo 2

Functional meat products enriched in carob fruit extract (Ceratonia siliqua, L.) in Type 2 Diabetes Mellitus prevention and treatment

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Adrián Macho González

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Madrid

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DEPARTAMENTO DE NUTRICIÓN Y CIENCIA DE LOS ALIMENTOS

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El **Dr. Francisco José Sánchez Muniz**, Catedrático del Departamento de Nutrición y Ciencia de los Alimentos de la Facultad de Farmacia de la Universidad Complutense de Madrid, la **Dra. Sara Bastida Codina**, Profesora Titular del Departamento de Nutrición y Ciencia de los Alimentos de la Facultad de Farmacia de la Universidad Complutense de Madrid, y la **Dra. Alba Garcimartín Álvarez**, Profesora Ayudante Doctor del Departamento de Farmacología, Farmacognosia y Botánica de la Facultad de Farmacia de la Universidad Complutense de Madrid,

CERTIFICAN que el trabajo de investigación titulado

"Cárnicos funcionales enriquecidos en extracto de algarroba (*Ceratonia siliqua*, L.) en la prevención y tratamiento de la Diabetes Mellitus tipo 2"

constituye la memoria que presenta el Licenciado **Adrián Macho González** para optar al grado de Doctor y ha sido realizado en los Departamentos de Nutrición y Ciencia de los Alimentos y de Farmacología, Farmacognosia y Botánica, ambos de la Facultad de Farmacia de la Universidad Complutense de Madrid, bajo nuestra dirección. Asimismo, en el marco del proceso de evaluación requerido, damos nuestro consentimiento para su presentación y defensa en la Universidad Complutense de Madrid.

Para que conste a los efectos oportunos, firmamos el presente certificado en Madrid a treinta y uno de mayo de dos mil veintiuno.

Francisco José Sánchez Muniz	Sara Bastida Codina	Alba Garcimartín Álvarez

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El trabajo de investigación que ha dado origen a esta memoria ha sido realizado en los Departamentos de Nutrición y Ciencia de los Alimentos y Farmacología, Farmacognosia y Botánica, ambos pertenecientes a la Facultad de Farmacia de la Universidad Complutense de Madrid, al amparo de los proyectos AGL 2014-53207-C2-2-R del Ministerio de Economía y Competitividad, PR75/18-21603 de los Proyectos Santander-UCM y PID2019-103872RB-I00 del Ministerio de Ciencia, Innovación y Universidades.

Asimismo, el doctorando D. Adrián Macho González ha disfrutado de una beca de Formación de Profesorado Universitario (FPU), referencia FPU15/02759, concedida por el Ministerio de Educación, Cultura y Deporte.

El doctorando ha disfrutado de una Estancia Breve (3 meses) con referencia EST17/00509, concedida por el Ministerio de Ciencia, Innovación y Universidades en el Instituto de Ciencia y Tecnología de los Alimentos y Nutrición (CSIC-ICTAN), bajo la supervisión de la Dra. Ascensión Marcos. También ha disfrutado de un Traslado Temporal (6 meses) con referencia EST18/00685 financiado por el Ministerio de Educación, Cultura y Deporte en el Albert Einstein College of Medicine de Nueva York, bajo la tutoría de la Dra. Ana María Cuervo.

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Índice

Resumen ... 19
Abstract ... 21
Índice de tablas ... 23
Índice de figuras ... 24
I. INTRODUCCIÓN ... 29
1. Diabetes Mellitus ... 30
1.1. Concepto y clasificación de la Diabetes Mellitus ... 31
1.2. Bases moleculares de las acciones de la insulina ... 34
1.2.1. Síntesis de insulina ... 35
1.2.2. Regulación de la secreción de insulina ... 35
1.2.3. Señalización de insulina ... 36
2. Diabetes Mellitus tipo 2 ... 38
2.1. Fisiopatología de la resistencia a la insulina ... 39
2.1.1. Defectos en la señalización InsR/IRS/PI3K/AKT ... 40
2.1.1. Estrés oxidativo como inductor de resistencia a la insulina ... 42
2.2. Dislipemia diabética asociada a la DMT2 ... 45
2.3. Enfermedad de hígado graso no alcohólico ... 47
2.4. Microbiota intestinal y DMT2 ... 48
2.4.1. Producción de AGCC y su implicación en la DMT2 ... 50
2.4.2. Modulación del metabolismo de la glucosa por la microbiota ... 51
2.4.3. Microbiota intestinal, integridad de barrera e inflamación ... 53
3. Algarroba ... 54
3.1. Fibra dietética como componente principal de la pulpa de algarroba. ... 56
3.2. Polifenoles asociados a la pulpa de la vaina de algarroba. ... 57
3.2.1. Proantocianidinas o taninos condensados ... 59
3.3. Beneficios para la salud derivados del consumo de algarroba ... 62
3.3.1. Efecto antioxidante de la algarroba y sus productos derivados ... 63
3.3.2. Efecto anticancerígeno de la algarroba y sus productos derivados ... 63
3.3.3. Efecto antidiarreico de la algarroba y sus productos derivados ... 64
3.3.4. Efecto antidiabético de la algarroba y sus productos derivados ... 64
3.3.5. Efecto hipolipemiante de la algarroba y sus productos derivados ... 65
4. La dieta como tratamiento para las enfermedades crónicas ... 66
4.1. Intervención nutricional en la DMT2 ... 67

4.2. Importancia de la carne en la dieta ... 71
4.3. Alimentos funcionales ... 72
4.4. Productos cárnicos como alimentos funcionales ... 75
4.5. Extracto de algarroba como ingrediente funcional ... 76
II. HIPÓTESIS Y OBJETIVOS ... 79
2.1. Interés del estudio ... 81
2.2. Hipótesis y objetivos ... 82
III. MATERIAL Y MÉTODOS ... 85
3.1. Proceso de obtención del Extracto de Algarroba ... 87
3.2. Técnicas *in vitro* ... 88
3.3. Técnicas *in vivo* ... 91
3.3.1. Diseño experimental del estudio *in vivo* a corto plazo ... 91
3.3.2. Formulación de los reestructurados cárnicos ... 91
3.3.3. Dietas empleadas en el estudio *in vivo* crónico ... 92
3.3.4. Diseño experimental del estudio *in vivo* crónico ... 94
3.3.4.1. Modelo murino de estadio inicial de DMT2 ... 94
3.3.4.2. Modelo murino de estadio tardío de DMT2 ... 94
3.3.4.3. Determinaciones plasmáticas ... 96
3.3.4.4. Western Blot ... 99
3.3.4.5. Inmunohistoquímica ... 100
3.3.4.6. Estudio histológico ... 101
3.3.4.7. Peroxidación lipídica ... 101
3.3.4.8. Determinación del glucógeno hepático ... 102
3.3.4.9. Valoración de la apoptosis ... 102
3.3.4.10. Extracción de ADN fecal. Análisis de la microbiota (qPCR). ... 102
3.3.4.11. Determinación de Ácidos Grasos de Cadena Corta ... 103
3.3.4.12. Análisis estadístico ... 104
IV. RESULTADOS ... 105
Capítulo 1 ... 107
Publicación 1 ... 109
Publicación 2 ... 119
Publicación 3 ... 129
Capítulo 2 ... 151
Publicación 4 ... 153
Publicación 5 ... 177

Publicación 6 205
Capítulo 3 229
Publicación 7 231
Publicación 8 243
Publicación 9 261
Publicación 10 275
Publicación 11 289
V. DISCUSIÓN INTEGRADORA 329
5.1. Efecto a corto plazo del consumo de extracto de algarroba sobre la digestión y absorción de hidratos de carbono y grasas 331
5.2. Impacto del consumo un cárnico enriquecido en CFE sobre la fisiopatología de dos modelos murinos de DMT2 337
5.2.1. Caracterización y justificación de idoneidad de los modelos animales empleados en los estudios crónicos 338
5.2.2. Impacto del consumo de un cárnico enriquecido en CFE en un modelo murino de estadio inicial de DMT2 (Artículo 7) 345
5.2.3. Impacto del consumo de un cárnico enriquecido en CFE en un modelo murino de estadio avanzado de DMT2. ¿Estrategia preventiva o terapéutica? (Artículos 8-11) 347
VI. RESUMEN Y CONCLUSIONES 357
VII. SUMMARY AND CONCLUSIONS 363
VIII. BIBLIOGRAFÍA 369

Resumen

Introducción: La Diabetes Mellitus tipo 2 (DMT2) es una patología crónica en la que la modificación de los hábitos de vida, particularmente los relacionados con la dieta, siguen siendo el pilar fundamental del tratamiento. El consumo de compuestos bioactivos de origen natural ha demostrado ser una estrategia nutricional efectiva para conseguir un mejor control glucémico y lipémico, lo que minimiza el impacto de la DMT2, su progresión y sus complicaciones en la salud.

El algarrobo (*Ceratonia siliqua*, L.) es un árbol de la familia de las fabáceas muy extendido en la zona Mediterránea, cuyo fruto ha demostrado tener numerosas aplicaciones tecnológicas y propiedades nutricionales y beneficiosas interesantes. De hecho, existe evidencia científica que relaciona su consumo con una mejora de las enfermedades crónico-degenerativas más prevalentes, como es el caso de DMT2. Estas mejoras se deben fundamentalmente a su alto contenido en fibra y proantocianidinas (PACs) responsables de sus efectos antioxidantes, anticancerígenos, hipolipemiantes y antidiabéticos.

Objetivo: Evaluar la eficacia de un extracto de algarroba (CFE, de sus siglas en inglés *Carob Fruit Extract*) como ingrediente funcional para formular productos cárnicos con propiedades antidiabéticas, así como caracterizar sus efectos sobre marcadores de la DMT2 y los principales mecanismos responsables de los mismos.

Material y métodos: Para responder al objetivo principal de la presente Tesis Doctoral se han llevado a cabo diferentes experimentos en ratas Wistar. El primero de ellos en animales jóvenes y sanos, para verificar los efectos del CFE sobre la digestión y absorción de hidratos de carbono y grasas. Posteriormente, se han empleado dos modelos de DMT2, que corresponden a los estadios inicial y avanzado de la patología, en los que se evalúan los efectos beneficiosos del consumo crónico de un cárnico funcional enriquecido en CFE.

Resultados: El CFE redujo la digestión y absorción de los hidratos de carbono y las grasas, al inhibir de forma dosis-dependiente la actividad de las enzimas α-glucosidasa (actividad maltasa) y lipasa pancreática; incrementar la excreción de macronutrientes en heces y reducir la expresión génica de transportadores clave como el SGLT1, responsable de la absorción de glucosa. Además, presentó una adecuada viabilidad tecnológica al emplearse en sistemas basados en emulsiones, manteniendo sus propiedades antioxidantes e inhibitorias de la lipasa pancreática. El consumo de un cárnico conteniendo CFE en el estadio inicial de la DMT2 preservó la sensibilidad a

la insulina en el hígado a través de la vía de señalización InsR/IRS/PI3K/AKT, lo que se tradujo en una menor hiperinsulinemia y dislipemia diabética. En cambio, su eficacia antidiabética en un estadio tardío de la patología está condicionado a si se consume de forma preventiva o terapéutica. El consumo del cárnico funcional de forma preventiva evitó la progresión de la patología, mostrando una mayor funcionalidad pancreática y sensibilidad a la insulina en el hígado, menor dislipemia y esteatosis hepática; y menor disbiosis, estrés oxidativo y permeabilidad colónica. En cambio, su consumo como tratamiento terapéutico revirtió y ralentizó la progresión de la patología al mantener una mayor sensibilidad hepática a la insulina, menor disfunción pancreática y esteatosis hepática, y mejor estatus antioxidante colónico.

Conclusión: El consumo del cárnico enriquecido en CFE ejerce un efecto antidiabético multidiana, reduciendo la glucemia y la lipemia, protegiendo la funcionalidad del páncreas y el hígado, mejorando el estatus antioxidante, la disbiosis y la integridad de la barrera colónica; aspectos que sugieren ampliamente que se trata de un alimento que mejora funcionalidades a nivel colónico, hepático y pancreático; y minimiza el riesgo de desarrollar DMT2.

Abstract

Introduction: Type 2 Diabetes Mellitus (T2DM) is a chronic pathology in which lifestyle habit modification, particularly those related to diet, continue to be the fundamental pillar of treatment. The consumption of natural origin bioactive compounds has proven to be an effective nutritional strategy to achieve better glycemic and lipemic control, which minimizes the impact of T2DM and its health complications.

The carob tree (*Ceratonia siliqua*, L.) is a tree in the Fabaceae family that is widespread in the Mediterranean area, whose fruit has shown numerous technological and nutritional applications. In fact, there is scientific evidence that links its consumption, to an improvement in the most prevalent chronic-degenerative diseases in developed countries, such as T2DM. These improvements are mainly due to its high content of fiber and proanthocyanidins (PACs) responsible its antioxidant, anticancer, lipid-lowering, and antidiabetic effects.

Objective: To evaluate the efficacy of a Carob Fruit Extract (CFE) as a functional ingredient to formulate meat products with antidiabetic properties, as well as to characterize its effects on T2DM markers and the main mechanisms responsible for them.

Materials and methods: To respond to the main objective of this Doctoral Thesis, different experiments have been carried out on Wistar rats. The first of them was carried out in young and healthy animals, to verify the CFE effects on the digestion and absorption of carbohydrates and fats. Subsequently, two models of T2DM were used, corresponding to the initial and late-stages of the disease, in which the beneficial effects of chronic consumption of CFE-enriched meat were evaluated.

Results: CFE consumption reduced the digestion and absorption of carbohydrates and fats, by inhibiting in a dose-dependent manner the activity of the α-glucosidase (maltase activity) and pancreatic lipase enzymes; increase macronutrient fecal excretion and reduce the gene expression of key transporters such as SGLT1. In addition, it presented adequate technological viability when used in emulsion-based systems, maintaining its antioxidant and inhibitory pancreatic lipase properties. The consumption of CFE-enriched meat in early-stage T2DM as a preventive strategy maintains insulin sensitivity in the liver through the InsR/IRS/PI3K/AKT signaling pathway, which translates into lower hyperinsulinemia and diabetic dyslipidemia. On the other hand, its antidiabetic efficacy in a late stage T2DM is conditioned to whether it is consumed preventively or therapeutically. The preventive consumption of CFE-

enriched meat blocked the progression of the pathology, showing greater pancreatic functionality and insulin sensitivity in the liver, less dyslipidemia, and hepatic steatosis, as well as less dysbiosis, oxidative stress, and colonic permeability. On the other hand, its use as a therapeutic treatment reversed and slowed down the progression of the pathology by maintaining greater hepatic sensitivity to insulin, less pancreatic dysfunction and hepatic steatosis, and better colonic antioxidant status.

Conclusion: The consumption of CFE-enriched meat exerts a multi-target antidiabetic effect, reducing glycemia and lipemia, protecting the functionality of the pancreas and liver, and improving the antioxidant status, dysbiosis, and the integrity of the colonic barrier, aspects widely suggest that CFE-enriched meat improves functionalities at the colonic, liver and pancreatic levels; and minimizes the risk of developing T2DM.

Índice de tablas

Tabla 1. Cambios en la microbiota asociados a la DMT2 49

Tabla 2. AGCC e implicación sobre la salud. 52

Tabla 3. Principales polifenoles encontrados en la algarroba 58

Tabla 4. Patrones nutricionales con mayor evidencia sobre la prevención y control de la DMT2 69

Tabla 5. Comparativa de la composición nutricional por 100g de carne magra de vacuno y cerdo procedente de España y EE.UU. 72

Tabla 6. Composición de los reestructurados cárnicos 92

Tabla 7. Composición de las dietas experimentales para el experimento crónico 93

Tabla 8. Anticuerpos primarios empleados en Western Blot e inmunohistoquímica 100

Tabla 9. Primers usados en la determinación de la microbiota mediante qPCR. 103

Tabla 10. Parámetros plasmáticos para definir el estadio de la DMT2. 343

Índice de figuras

Figura 1. Prevalencia de adultos (20-79 años) con Diabetes a nivel mundial en 2019. ... 30

Figura 2. Bases moleculares de la secreción de insulina. ... 36

Figura 3. Vía inhibitoria de la translocación de GLUT-4 por ácidos grasos libres. ... 41

Figura 4. Esquema representativo de la fisiopatología de la DMT2 ... 44

Figura 5. Rutas principales del metabolismo lipoproteico. ... 46

Figura 6. Principales componentes de la pulpa y semilla de la algarroba. ... 56

Figura 7. Tipos de interacciones entre polifenoles y fibra dietética ... 57

Figura 8. Estructura química básica de las PACs y sus conformaciones monoméricas ... 59

Figura 9. Catabolismo microbiano de un dímero de procianidina B2 ... 61

Figura 10. Efectos beneficiosos del consumo de PACs en el organismo. ... 65

Figura 11. Principales estrategias a la hora de diseñar un alimento funcional ... 74

Figura 12. Diseño experimental del estudio *in vivo* a corto plazo. ... 91

Figura 13. Diseño experimental del estudio *in vivo* en un modelo murino de estado inicial de DMT2 ... 94

Figura 14. Diseño experimental del estudio *in vivo* en un modelo murino de estadio tardío de DMT2 ... 95

Figura 15. Estructuras de las PACs identificadas en el CFE. ... 332

Figura 16. Efecto del consumo del CFE sobre la glucemia postprandial. ... 333

Figura 17. Efecto del consumo del CFE sobre la trigliceridemia postprandial. ... 334

Figura 18. Esquema representativo del efecto del CFE sobre la digestión y absorción de hidratos de carbono y grasas. ... 337

Figura 19. Progresión de la DMT2 en relación con la funcionalidad de las células β pancreáticas. DMT2, Diabetes Mellitus tipo 2 ... 339

Figura 20. Comparativa entre los grupos control pertenecientes al estadio inicial y tardío de DMT2. ... 342

Figura 21. Cambios inducidos por el consumo del cárnico enriquecido en CFE en el grupo CE en comparación con el control C. ... 346

Figura 22. Efecto del consumo crónico del cárnico enriquecido en CFE sobre la disfunción pancreática. ... 348

Figura 23. Efecto del consumo del cárnico enriquecido en CFE sobre la morfología colónica. 351

Figura 24. Cambios inducidos por el consumo del cárnico enriquecido en CFE en comparación con el control D. 354

Abreviaturas

ADA	Asociación Americana de Diabetes
AGCC	Ácidos grasos de cadena corta
AGE	Productos finales de glicosilación avanzados
AGL	Ácidos grasos libres
AGM	Ácidos grasos monoinsaturados
AGP	Ácidos grasos poliinsaturados
AGS	Ácidos grasos saturados
Apo	Apolipoproteína
ATP	Adenosín trifosfato
CAT	Cattalasa
ChREBP	Proteína de unión al elemento de respuesta a carbohidratos
CFE	Extracto de algarroba
DM	Diabetes Mellitus
DMG	Diabetes Mellitus Gestacional
DMT1	Diabetes Mellitus tipo 1
DMT2	Diabetes Mellitus tipo 2
DNL	Lipogénesis *de novo*
ECV	Enfermedades cardiovasculares
GIP	Polipéptido inhibidor gástrico
GLP-1	Péptido similar al glucagón tipo 1
GLUT-2	Transportador de glucosa tipo 2
GLUT-4	Transportador de glucosa tipo 4
GPR	Receptor para ácidos grasos de cadena corta
GPx	Glutation peroxidasa
GR	Glutation reductasa
GS	Glucógeno sintasa
GSK3	Glucógeno sintasa quinasa 3
GWAS	Estudio de asociación del genoma completo
HbA1c	Hemoglobina glicosilada
HDL	Lipoproteínas de alta densidad
HOMA-IR	Modelo homeostático de resistencia a la insulina

HOMA-β	Modelo homeostático de funcionalidad de células β pancreáticas
IDF	Federación Internacional de Diabetes
IDL	Lipoproteínas de densidad intermedia
InsR	Receptor de insulina
IRS	Sustrato del receptor de insulina
LADA	Diabetes autoinmune latente del adulto
LDL	Lipoproteínas de baja densidad
LDLr	Receptor para LDL
LSH	Lipasa sensible a hormonas
LXR	Receptor X hepático
MDA	Malondialdehído
NAD	Nicotinamida
NAFLD	Enfermedad de hígado graso no alcohólico
NASH	Esteatohepatitis no alcohólica
OMS	Organización Mundial de la Salud
PACs	Proantocianidinas
p.c.	Peso corporal
PDK	Quinasa dependiente de fosfolípidos
PI3K	Fosfatidilinositol 3-quinasa
PYY	Péptido YY
RI	Resistencia a la Insulina
RNS	Especies reactivas de nitrógeno
ROS	Especies reactivas de oxígeno
SGLT1	Cotransportador de sodio-glucosa tipo 1
SOD	Superóxido dismutasa
SREBP-1c	Proteína de unión al elemento regulador del esterol 1c
TBARS	Sustancias reactivas al ácido tiobarbitúrico (TBARS)
STZ	Estreptozotocina
VLDL	Lipoproteínas de muy baja densidad

I. INTRODUCCIÓN

1. Diabetes Mellitus

La Diabetes Mellitus (DM) es una patología crónica caracterizada por niveles elevados de glucosa en sangre como consecuencia de una producción insuficiente de insulina, o como señala la Federación Internacional de Diabetes (IDF por sus siglas en inglés, *International Diabetes Federation*), por deficiencias en el mecanismo de transducción de señales de dicha hormona en los tejidos diana (IDF, 2019). La DM es una de las diez principales causas de muerte a nivel mundial, considerándose una de las mayores emergencias sanitarias del siglo XXI (Zimmet y cols., 2016). Se calcula que aproximadamente 4,2 millones de adultos de entre 20 y 79 años han fallecido como resultado de la diabetes y sus complicaciones en 2019. Su elevada prevalencia estima que 463 millones de personas en todo el mundo, o el 9,3% de los adultos de 20 a 79 años, padecen DM, lo cual supone un gran riesgo sanitario. Además, las estadísticas sugieren que los datos van a continuar aumentando con el paso de los años, ya que se prevé que la cantidad total aumente hasta 578 millones (+10,2%) para 2030 y hasta 700 millones (+10,9%) para 2045 (Figura 1). A pesar de que estos datos son abrumadores, la IDF estima que aproximadamente el 50% de las personas con DM no están diagnosticadas (IDF, 2019).

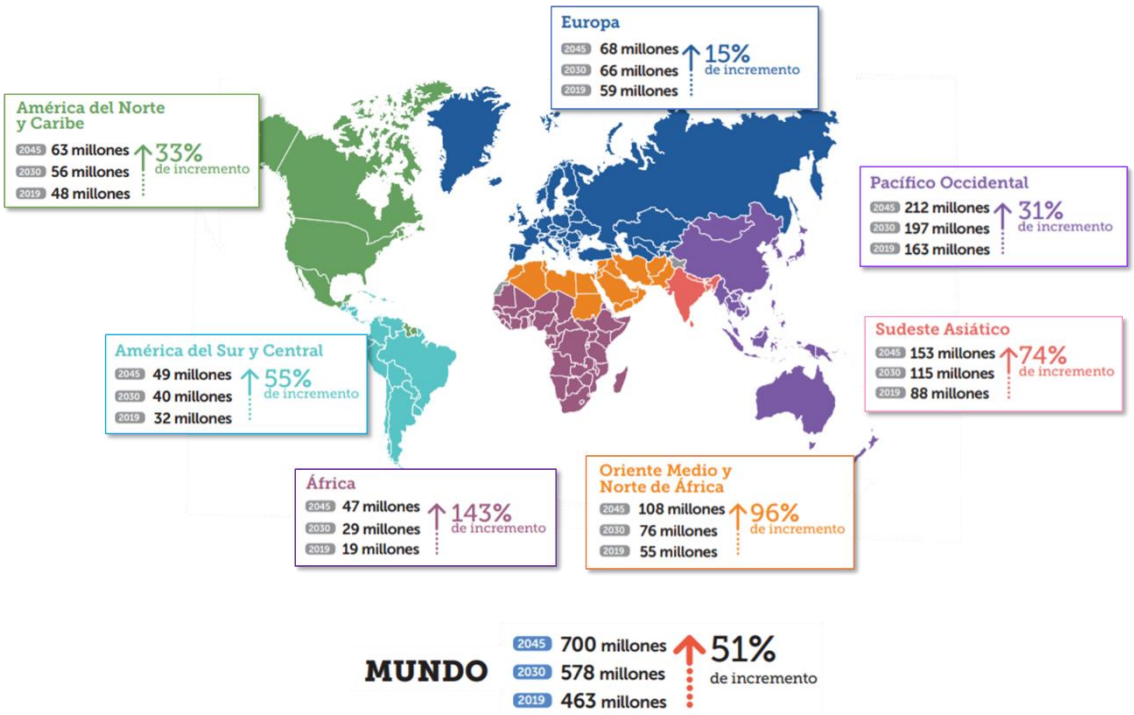

Figura 1. Prevalencia de adultos (20-79 años) con Diabetes a nivel mundial en 2019.
Los porcentajes son indicativos del aumento de la prevalencia estimado para el año 2045.
Modificado del Atlas de la Diabetes de la IDF (Novena Edición)

Teniendo en cuenta las cifras anteriormente descritas, es importante destacar que la Diabetes Mellitus tipo 2 (DMT2) es la principal causante de la epidemia de DM a nivel mundial, representando aproximadamente el 90% de los casos de DM en todo el mundo (IDF, 2019). Esta patología se asocia con hábitos de vida sedentarios y una alimentación poco saludable, lo que ha originado un incremento exponencial de su prevalencia en los países más desarrollados. Sin embargo, la IDF pone el foco en todos aquelos países que han visto mejorada su economía de forma reciente, donde la transición nutricional puede contribuir a un mayor aumento de la prevalencia (Figura 1).

1.1. Concepto y clasificación de la Diabetes Mellitus

A finales de noviembre de 1964, en un intento de lograr un consenso internacional sobre una clasificación de la DM, se elaboró el primer informe del Comité de Expertos de la Organización Mundial de la Salud (OMS) sobre la DM (McDonald y cols., 1965). Esta primera reunión apenas tuvo repercusión, siendo el segundo Comité de Expertos de la OMS sobre diabetes, apoyado en lo establecido por el *National Diabetes Data Group* un año antes, quien estableció las diferentes categorías de diabetes e intolerancia a la glucosa (Harris, 1979; WHO, 1980). Posteriormente, se han ido realizando modificaciones y puntualizaciones a los criterios de clasificación, estableciendo finalmente un valor de glucosa en ayunas para el diagnóstico de diabetes de ≥126 mg/dL o ≥7mmol/L. Asimismo, también se introdujo una nueva categoría para el metabolismo anormal de la glucosa denominada glucemia alterada en ayunas, para la que se establece un intervalo de glucosa en sangre de 100-125 mg/dL o 5,6 a 7,0 mmol/L, respectivamente (Alberti & Zimmet, 1998). Además de la medición directa de los niveles de glucosa en el plasma como método diagnóstico, la DM suele ir acompañada de una serie de síntomas como polidipsia, poliuria, polifagia, visión borrosa y pérdida de peso. El deterioro del crecimiento y la susceptibilidad a ciertas infecciones también pueden acompañar a la hiperglucemia crónica. Las consecuencias agudas y potencialmente mortales de la DM no controlada son la hiperglucemia con cetoacidosis o el síndrome hiperosmolar no cetósico (American Diabetetes Association, 2014a). Además, la DM presenta grandes complicaciones macro y microvasculares a largo plazo, siendo una de las principales causas de mortalidad asociadas a dicha patología. En relación con las complicaciones diabéticas microvasculares fundamentalmente recogen a la nefropatía diabética que conduce a insuficiencia renal; a la neuropatía periférica con riesgo de úlceras en los pies, amputaciones y articulaciones de Charcot; y a la retinopatía con pérdida potencial de

visión. Por otra parte, las macrovasculares contemplan la enfermedad coronaria, enfermedad arterial periférica e infarto de miocardio (Maric-Bilkan, 2017).

La formá más habitual de clasificar la DM se basa en su etiopatogenia (American Diabetetes Association, 2014a), encontrándose los siguientes tipos:

1. **Diabetes Mellitus Tipo 1 (DMT1)**. Es una enfermedad crónica de origen autoinmune que se caracteriza por la destrucción de las células β pancreáticas y la consiguiente hiperglucemia por déficit de insulina (Katsarou y cols., 2017). Esta enfermedad puede desarrollarse a cualquier edad, pero suele aparecer más frecuentemente en niños o adolescentes, con una mayor incidencia entre los 10 y 14 años. Apenas representa el 10% de los casos de DM a nivel mundial, pero su incidencia se está incrementando notablemente (IDF, 2019). Las personas con DMT1 necesitan la administración de un análogo de insulina para poder mantener la homeostasis de la glucosa, siendo éste el principal tratamiento. El control glucémico óptimo requiere dosis múltiples de insulina, de forma que se imite la liberación fisiológica de esta hormona, aportando insulina al inicio para el control durante la noche e interprandial, junto con dosis en bolo (Kahanovitz y cols., 2017).

2. **Diabetes Mellitus Tipo 2 (DMT2)**. Se caracteriza por hiperglucemia como resultado de la incapacidad de las células del organismo de responder a la insulina, lo que se conoce como RI (IDF, 2019). Durante este estado existe una alteración en el mecanismo de transducción de señales de la insulina que provoca una menor respuesta en las células diana, efecto que se contrarresta por un aumento en la secreción de dicha hormona que consigue mantener la homeostasis de la glucosa. En etapas avanzadas, la producción de insulina disminuye como consecuencia de un agotamiento de la célula β pancreática. Esta patología representa entre el 90-95% de los casos de DM en la actualidad, con unas cifras de 417-440 millones de personas afectadas a nivel mundial. La mayor prevalencia de este tipo de DM se observa en personas mayores de 45, pero cada vez es más frecuente en niños, adolescentes y adultos más jóvenes, debido a los niveles crecientes de obesidad, inactividad física y dietas hipercalóricas desde edades tempranas.

3. **Diabetes Mellitus Gestacional (DMG)**. Se define como intolerancia a los hidratos de carbono con inicio o primera detección durante el embarazo. Asimismo, la ADA la define como "diabetes diagnosticada durante el embarazo que no es claramente una diabetes manifiesta" (American Diabetetes

Association, 2014b). Es importante diferenciar la DMG, en la que los valores de glucemia son ligeramente elevados y que se normalizan tras el parto, de la diabetes en el embarazo, cuyas cifras son mucho más elevadas y no se resuelve una vez finalizada la etapa de gestación (McIntyre y cols., 2019). Se calcula que aproximadamente la mayoría de los casos (75-90%) de hiperglucemia durante el embarazo son DMG (IDF, 2019). La DMG es una de las complicaciones médicas más frecuentes durante el embarazo, afectando aproximadamente al 14% de las embarazadas a nivel mundial. Gesteiro et al. (Gesteiro y cols., 2009) encontraron en una población española de mujeres embarazadas del estudio Mérida una prevalencia del orden del 11% de DMG.

4. **Otros tipos específicos de DM**. Se engloban en este grupo la tipo LADA (Diabetes autoinmune latente del adulto), anormalidades en el metabolismo de la glucosa debidas a otras causas, como defectos genéticos en la función de las células β y/o en la acción de la insulina, enfermedades del páncreas exocrino (pancreatitis, neoplasia, fibrosis quística, etc.), endocrinopatías (acromegalia, síndrome de Cushing, etc.), uso de drogas, medicamentos o componentes químicos (glucocorticoides, ácido nicotínico, etc.), infecciones (citomegalovirus) y otros síndromes asociados en ocasiones a la DM (síndrome de Down, de Klinefelter, de Turner, etc.)
    - LADA: es un tipo de DM autoinmune que comienza en la edad adulta y no necesita insulina para el control glucémico, al menos en los primeros seis meses después del diagnóstico. Para poder enmarcar a los pacientes en este tipo, es necesario que cumplan: ser adultos mayores de 35 años, tener autoanticuerpos positivos contra las células β pancreáticas y no ser insulinodependentes durante al menos los seis primeros meses (Pieralice & Pozzilli, 2018).
    - Defectos genéticos de la célula β: están asociados con defectos monogénicos en la célula β que cursan con alteración en su funcionalidad. Comúnmente, la hiperglucemia suele tener un inicio temprano, antes de los 25 años, como consecuencia de una secreción de insulina alterada con defectos mínimos o nulos en la acción de dicha hormona. Reciben el nombre de tipo MODY (de sus siglas en inglés, *maturity-onset diabetes of the young*).
    - Defectos genéticos en la acción de la insulina: representan una baja prevalencia y se deben a anormalidades genéticamente determinadas en alguna de las etapas de la señalización de insulina. La más común es la

mutación en el receptor de insulina, cuya afección produce hiperinsulinemia e hiperglucemia moderada o severa.

- Enfermedades del páncreas exocrino: cualquier lesión que se produzca en el páncreas y comprometa la secreción o síntesis de insulina puede causar diabetes. Las alteraciones adquiridas incluyen pancreatitis, infecciones, pancreatectomía y cáncer de páncreas.
- Endocrinopatías: la producción excesiva de hormonas que antagonicen la acción de la insulina puede inducir diabetes. Algunas de las patologías más comunes que se asocian a la diabetes son la acromegalia (por exceso de hormona de crecimiento), síndrome de Cushing (cortisol), glucagonoma (glucagón) o feocromocitoma (catecolaminas), entre otros.
- Diabetes inducida por fármacos o químicos: numerosos compuestos químicos/drogas pueden afectar la síntesis y secreción de insulina produciendo diabetes. En la actualidad son pocos los casos que se recogen, pero es posible que algunos compuestos como vacor o pentamidina intravenosa destruyan a las células β pancreáticas; mientras que otros como el ácido nicotínico, glucocorticoides, hormona tiroidea o interferón α, pueden precipitar a la diabetes en personas con resistencia a la insulina.

1.2. Bases moleculares de las acciones de la insulina

La insulina (del latín *insula*, "isla") es una hormona anabolizante producida por las células β del páncreas endocrino que controla la conservación y utilización de energía durante los estados de alimentación y ayuno. Frederick Grant Banting y Charles Best, supervisados por John James Richard MacLeod, fueron quienes descubrieron la insulina en 1921. Este descubrimiento supuso un hito en el campo de la medicina y les llevó a recoger el Premio Nobel de Fisiología o Medicina en 1923 (Vecchio y cols., 2018).

Como se ha mencionado, la insulina es la principal hormona anabólica del organismo y su síntesis, control de calidad, transporte y acción están regulados estrechamente. Estas funciones se realizan mediante mecanismos intracelulares altamente conservados que comienzan en la célula β del páncreas, continúan con su entrega y acción en los diferentes tejidos, y finalizan con su degradación en el riñón. Para poder conocer a fondo este “viaje” de la insulina, a continuación se describen en detalle los procesos de síntesis, secreción y señalización.

1.2.1. Síntesis de insulina

El gen humano de la insulina situado en el brazo corto del cromosoma 11 (11p15.5) codifica una proteína de 110 aminoácidos, conocida como preproinsulina. Como otras proteínas de secreción, la secuencia de la preproinsulina contiene en su extremo N-terminal hidrófobo al péptido señal, cuya interacción con las denominadas ribonucleoproteínas citosólicas de reconocimiento de señal, facilita la translocación de la molécula a través del retículo endoplásmico y la activación de la peptidasa señal que convierte la preproinsulina en proinsulina, de 81 aminoácidos. La proinsulina adopta la estructura terciaria mediante el plegamiento de la molécula y la formación de tres enlaces disulfuro mediada por chaperonas y por la proteína disulfuro isomerasa. Posteriormente, la proinsulina plegada es transportada desde el retículo endoplásmico al aparato de Golgi, donde se introduce en las vesículas secretoras. La activación de peptidasas específicas induce la escisión de la molécula, originando la insulina monomérica activa y el péptido C o péptido conector, de 30 aminoácidos, los cuales quedan almacenados en dicho orgánulo. Tras la acción de las peptidasas, dos de los tres puentes disulfuro se convierten en intercatenarios uniendo las cisteínas A7-B7 y A20-B19, respectivamente, mientras que el tercero une dos cisteínas de la cadena A (A7-A11). Estos enlaces contribuyen a la estabilidad de la estructura de la insulina, a la vez que determinan la afinidad por su receptor en los tejidos diana (De Meyts, 2004). La insulina almacenada en las células β se empaqueta en gránulos densamente agrupados, adoptando dicha molécula una estructura cuaternaria hexamérica, dispuesta como tres dímeros, que no es funcional, pero que resulta favorecida a concentraciones elevadas de hormona y permite su almacenamiento de forma más rentable (Weiss y cols., 2000).

1.2.2. Regulación de la secreción de insulina

La secreción de insulina está regulada principalmente por la cantidad de glucosa presente en el torrente sanguíneo, aunque también puede ser estimulada por la presencia de aminoácidos. Tras la ingesta de alimentos, la glucosa pasa al interior de la célula β mediante el transportador GLUT-2, para inmediatamente ser fosforilada en posición 6 por la hexoquinasa IV, más conocida como glucoquinasa, iniciando así su degradación por la vía glucolítica hasta piruvato. GLUT-2 y glucoquinasa, debido a sus elevadas Km, son dos sensores de glucosa ampliamente conocidos presentes también en hígado y en ciertas regiones del hipotálamo. La utilización del piruvato como fuente de acetil-CoA y la descarboxilación del acetato en el ciclo de Krebs proporciona el poder reductor necesario para la síntesis de adenosín trifosfato (ATP). El aumento de

la relación ATP/ADP en el citosol celular induce el cierre de los canales de potasio (K^+) sensibles a ATP. La consiguiente despolarización de la membrana plasmática origina la apertura de los canales de calcio (Ca^{2+}) sensibles a voltaje. El aumento de la concentración intracelular de Ca^{2+} induce una cascada de señales que culmina con la fusión de las vesículas secretoras de insulina con la membrana (exocitosis) y la consecuente liberación de la hormona (Fu y cols., 2013). Cualquier compuesto, natural o farmacológico, que interactúe con este mecanismo se considerará, un estimulador o un inhibidor de la secreción de insulina inducida por glucosa. Así, todo compuesto que aumente las concentraciones de ATP, cierre los canales de potasio, abra los canales de calcio de la membrana plasmática o de los orgánulos subcelulares, o promueva la fusión de las vesículas de secreción con la membrana plasmática de las células β inducirá la secreción de insulina (Figura 2).

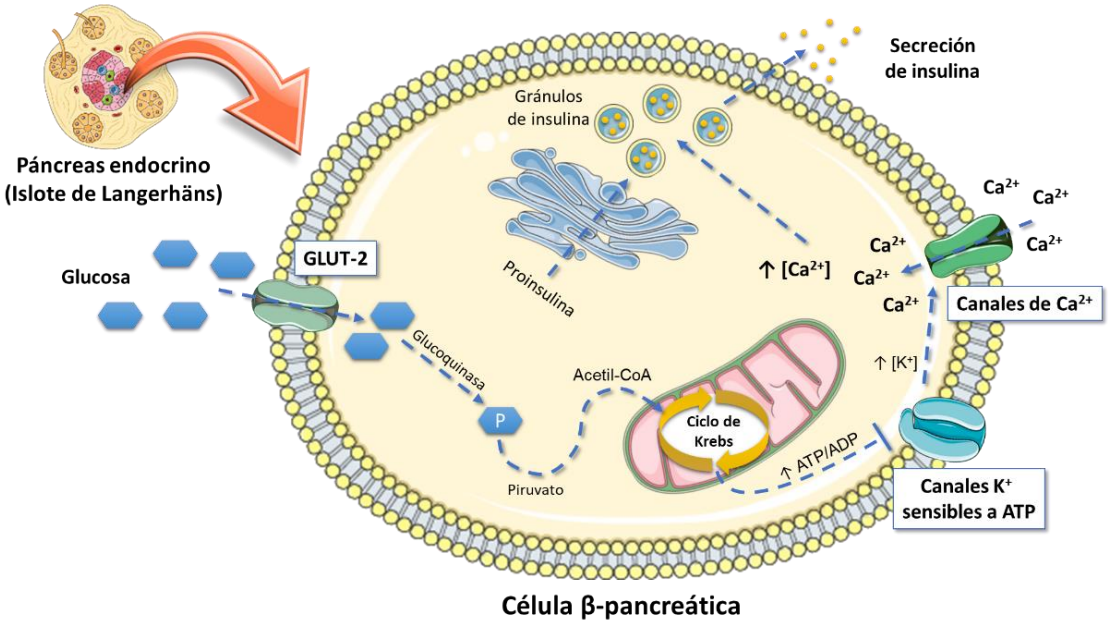

Figura 2. Bases moleculares de la secreción de insulina.

ADP, adenosín difosfato; ATP, adenosín trifosfato; Ca2+, calcio; GLUT-2, transportador de glucosa tipo 2; K+, potasio; P, glucosa-6-fosfato. Elaborado a partir de Fu y cols. (2013)

1.2.3. Señalización de insulina

El incremento de la glucemia plasmática tras la ingesta de alimentos, es rápidamente contrarrestado mediante la liberación de insulina por parte del páncreas. Para que este proceso tenga lugar, es necesario que la insulina se una a su receptor y se produzca una transducción de señales que conduzcan a la captación de glucosa por parte de los diferentes tejidos del organismo. Aunque la insulina estimula la captación de glucosa en muchos tipos de células, tiene efectos más pronunciados

sobre los adipocitos y las células musculares, donde es capaz de aumentar la captación de glucosa en aproximadamente diez y cinco veces, respectivamente (Lauro y cols., 1998). Cabe mencionar que cada paso en la cascada de señalización de insulina es una reacción enzimática reversible, es decir, por cada quinasa activada por insulina, existen fosfatasas que terminan su acción y regulan estrechamente su proceso.

El receptor de insulina (InsR) es un heterotetrámero con actividad intrínseca tirosina-quinasa que se localiza en la membrana plasmática y está compuesto por dos subunidades α y dos subunidades β, unidas por puentes disulfuro. Las subunidades α se localizan en el exterior de la membrana plasmática, contienen sitios de unión a la insulina y actúan como subunidades reguladoras; mientras que las subunidades β cruzan la membrana plasmática, presentando una porción extracelular, una transmembrana y una intracelular que contiene el dominio con actividad tirosina-quinasa que se autofosforila en los residuos Tyr^{1158}, Tyr^{1162} y Tyr^{1163}. Después de la activación del InsR por trifosforilación de su bucle de activación, éste fosforila a los sustratos del receptor de insulina (IRS), desencadenándose la transducción de señales a través de dos vías principales: ruta de la fosfatidilinositol 3-quinasa(PI3K)/AKT y vía de las quinasas activadas por mitógenos (MAP quinasas) (De Meyts, 2000).

- **Vía de PI3K/AKT:** es la principal ruta por el que la insulina ejerce sus funciones sobre el metabolismo de la glucosa y los lípidos. La transducción de señales se inicia con la fosforilación de IRS por parte de InsR, concretamente la isoforma 1, la cual se ha observado que es la predominante en la captación de glucosa estimulada por insulina. Esta activación de IRS, permite la unión y activación de proteínas con dominios SH2 (de homología al dominio 2 de la proteína Src) como PI3K (Haeusler y cols., 2018). La PI3K está conformada por una subunidad reguladora (p85) que presenta los dominios SH2 para su interacción con IRS, y una subunidad catalítica (p110), que se encarga de fosforilar el sustratos fosfatidilinositol-4,5-bisfosfato (PtdIns(4,5)P2 o PIP2) para generar la fosfatidilinositol-3,4,5-trifosfato (PIP3). Este fosfolípido actúa como segundo mensajero y recluta a la membrana plasmática a diferentes proteínas de señalización intracelular que presenten un dominio de homología de pleckstrina, el cual sirve de anclaje y permite la transmisión del mensaje al interior celular. Las quinasas dependientes de fosfolípidos (PDK1 y PDK2) o la proteína quinasa B (AKT/PKB) presentan dicho dominio, lo que permite su translocación a la membrana celular. PDK1 fosforila AKT en la treonina 308,

pero para su completa activación necesita la fosforilación en serina 473 mediada por mTORC2. AKT fosforilado se libera del complejo asociado a la membrana y se conduce al citoplasma, donde regula diferentes sustratos que propagan la respuesta a la insulina. Entre las funciones metabólicas mejor caracterizadas reguladas por AKT se puede destacar la activación de la síntesis de glucógeno al fosforilar e inactivar la enzima glucógeno sintasa quinasa 3 (GSK3), la translocación del transportador de glucosa (GLUT-4) en músculo y tejido adiposo, o la fosforilación del factor de transcripción involucrado en la gluconeogénesis (FoxO1).

- **Vía de MAP quinasas (Raf/Ras/MEK/MAPK):** esta segunda ruta presenta gran diversidad de sustratos potenciales y su activación es independiente de la vía PI3K/AKT. Tanto la autofosforilación del InsR como la activación de IRS promueven la asociación de la proteína Shc. Su posterior fosforilación en tirosina induce el reclutamiento de Grb2, que se asocia con SOS para activar la ruta Ras-Erk1/2. La activación de Ras (GTP-Ras) induce la unión y activación de Raf-1, que conduce a la fosforilación y activación de la vía MEK y las quinasas reguladas extracelularmente 1 y 2 (ERK 1 y 2). Su activación juega un papel directo en la proliferación y diferenciación celular, regulando la expresión de genes y eventos extracelulares como la organización del citoesqueleto (Gehart y cols., 2010).

2. Diabetes Mellitus tipo 2

El pilar básico que define la DMT2 es la RI, estado en el que los tejidos periféricos no son capaces de responder a la acción de la insulina. En esta condición patológica, las células son incapaces de captar la glucosa plasmática tras una comida, produciéndose un estado hiperglucémico. Ante esta situación, el páncreas incrementa la secreción de insulina para tratar de vencer esta barrera, lo que conduce a un circuito de retroalimentación que finaliza en el agotamiento de la célula β pancreática (Petersen & Shulman, 2018). Esta alteración podría tener un componente genético, tal y como se observó en un estudio de asociación del genoma completo (GWAS), donde encontraron más de 400 variantes genéticas asociadas a la DMT2, la gran mayoría relacionadas con la funcionalidad de los islotes pancreáticos (Mahajan y cols., 2018). Curiosamente, esta predisposición genética solo puede responder al 20% del riesgo general de la enfermedad, siendo los hábitos de vida poco saludables los principales factores de riesgo. Concretamente, el consumo elevado de carnes procesadas,

bebidas azucaradas y hierro hemo, junto con la inactividad física, se han asociado positivamente con el desarrollo de DMT2. En cambio, un patrón de alimentación saludable que incluya productos integrales en la dieta, ha demostrado una reducción del riesgo (Bellou y cols., 2018). Teniendo en cuenta estos datos, se puede confirmar que la modificación de los hábitos alimentarios es un pilar básico para paliar esta patología. Como se verá posteriormente, uno de los objetivos clave de esta Tesis Doctoral es aminorar los efectos lesivos de la DMT2 mediante la inclusión de ingredientes funcionales en la dieta.

Anteriormente señalábamos que en una etapa inicial de DMT2 las células β pancreáticas compensan la RI al secretar más insulina al torrente sanguíneo. Esta situación conduce a un estado de hiperinsulinemia que promueve la lipogénesis *de novo* hepática (DNL, por sus siglas en inglés *de novo lipogenesis*), esteatosis, hiperlipidemia y expansión del tejido adiposo blanco. A medida que el estado diabético avanza se produce una disfunción del tejido adiposo blanco como consecuencia de la RI y la inflamación, lo que lleva a estimulación de la lipolisis. Esta liberación continuada de ácidos grasos libres (AGL) agrava aún más la RI hepática y favorece el desarrollo de hígado graso no alcohólico (NAFLD, de sus siglas en inglés *non-alcoholic fatty liver disease*) (López-Oliva & Muñoz Martínez, 2014). A su vez, el aumento del flujo de AGL y/o glicerol al hígado estimula la gluconeogénesis, lo que combinado con la disfunción de las células β, conduce a la hiperglucemia tanto postprandial como en ayunas de los pacientes con DMT2 (Mahajan y cols., 2018). A continuación se describirán los mecanismos y alteraciones más comunes que se producen en la DMT2.

2.1. Fisiopatología de la resistencia a la insulina

La RI es un trastorno metabólico que desafía una sola vía etiológica y para la que todavía no se tiene una respuesta clara, por lo que es necesario intentar comprender las diferentes rutas y tejidos afectados para poder tener una visión global del problema. La evidencia actual señala a una actividad defectuosa en la vía InsR/IRS/PI3K/AKT anteriormente descrita como principal responsable de la RI (Samuel & Shulman, 2012). Sin embargo, la bibliografía también describe un conjunto de mecanismos asociados al estrés oxidativo que participan en el desarrollo de esta alteración. Por tanto, es necesario abordar estos dos mecanismos para intentar comprender la fisiopatología de la DMT2.

2.1.1. Defectos en la señalización InsR/IRS/PI3K/AKT

De acuerdo a lo descrito previamente, esta ruta es la más importante en la transducción de señales por la insulina. Comúnmente, cuando se habla de RI se hace referencia a tres tejidos como participantes principales de este problema, músculo, tejido adiposo e hígado.

Músculo: La función de la insulina en el músculo esquelético es promover la captación de glucosa celular a través de la translocación del transportador GLUT-4 a la membrana plasmática y la consiguiente síntesis de glucógeno mediante la glucógeno sintasa (GS) (Samuel & Shulman, 2012). Este proceso es uno de los componentes principales de la RI asociada a la obesidad y DMT2, ya que es el principal tejido para la eliminación de la glucosa plasmática estimulada por insulina. La gran mayoría de estudios han demostrado que la RI en el músculo esquelético se debe a una menor expresión o actividad quinasa de las proteínas implicadas en la transducción de señales (Fazakerley y cols., 2019). Esta afirmación se inició en trabajos en ratones y humanos obesos, donde se observaron menores niveles InsR y una actividad quinasa disminuida en receptores aislados (Caro y cols., 1987; Kolterman y cols., 1981). Es probable que la alteración encontrada en IRS, PI3K y AKT en modelos diabéticos pueda estar influenciada por los cambios encontrados a nivel de receptor, cuya dificultad en la interacción ligando-receptor y la consiguiente transducción de señales, justificaría la menor expresión de las proteínas de esta ruta (Petersen & Shulman, 2018). Curiosamente, el músculo esquelético no desarrolla RI a través de la señalización de MAPK (Cusi y cols., 2000).

Tejido adiposo: a pesar de que el músculo es el principal consumidor de glucosa tras una comida, estudios que han evaluado la progresión de la DMT2 en ratones alimentados con una dieta alta en grasas han demostrado que la RI del tejido adiposo precede a la del músculo esquelético (Fazakerley y cols., 2019). Los mecanimos implicados son muy similares a los descritos anteriormente, donde se ha observado una disminución de la expresión y activación de las proteínas implicadas en la ruta InsR/IRS/PI3K/AKT. Sin embargo, dado que el tejido adiposo es un órgano endocrino que juega un papel importante en la DMT2 al liberar adipocinas, factores proinflamatorios y ácidos grasos libres (AGL), cabe mencionar otros mecanimos que participan en el desarrollo de RI. Una de las acciones más importantes de la insulina sobre el tejido es la supresión de la lipolisis. No obstante, en el marco de la DMT2 el tejido adiposo se vuelve resistente a la acción antilipolítica de la insulina, y es incapaz de suprimir la hidrólisis de triglicéridos y la liberación de AGL. Esta liberación

exacerbada de AGL incrementa significativamente las concentraciones plasmáticas, deteriorando la señalización de insulina y promoviendo la gluconeogénesis hepática. Esta asociación fue descrita por en 1963 por Randle y col. al observar que las concentraciones elevadas de AGL se correlacionaban con anormalidades en el metabolismo de los hidratos de carbono (Randle y cols., 1963). A pesar de que no se conoce el mecanismo exacto de lipotoxicidad, la evidencia indica que el mecanismo que favorece la RI se debe a los restos lipídicos activos como el diacilglicerol, la ceramida o los acil-CoA en lugar del grupo triglicérido inerte (Karpe y cols., 2011). Estos metabolitos de AGL interaccionan con serina/treonina quinasas o citoquinas (PKC, IKK, NFkB, TNFα) capaces de inhibir IRS y AKT, disminuyendo de esta manera la señalización de insulina y la consiguiente translocación de GLUT-4 a la membrana en los diversos tejidos del organismo (Figura 3) (Mukherjee y cols., 2013). Es necesario resaltar que la liberación local de citoquinas (TNFα, IL-1β e IL-6) dentro del tejido adiposo puede ser suficiente para inducir RI adiposa sin efectos relacionados con la disfrunción de otros tejidos, lo que generalmente se asocia con la llamada inflamación subclínica o de bajo grado (Roden & Shulman, 2019).

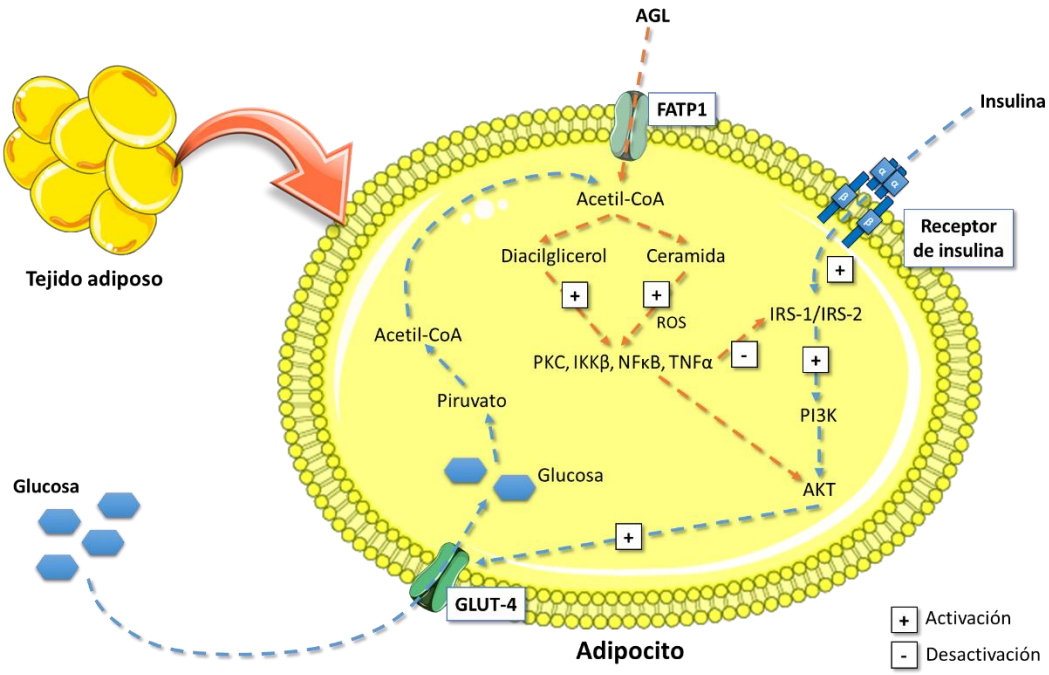

Figura 3. Vía inhibitoria de la translocación de GLUT-4 por ácidos grasos libres.

AGL, ácidos grasos libres; FATP1, proteína transportadora de ácidos grasos; GLUT-4, transportador de glucosa tipo 4; IKKβ, inhibidor del factor nuclear kappa B; IRS1/2, sustrato de insulina tipo 1/2; NFκB, factor nuclear kappa B; PI3K, fosfatidilinositol 3-quinasa; PKC, proteína quinasa C; ROS, especies reactivas de oxígeno; TNFα, factor de necrosis tumoral α. Elaborado a partir de Mukherjee y cols. (2013)

Hígado: órgano que juega un papel clave en la regulación de la homeostasis energética en todo el organismo. Una de las principales funciones de la insulina a nivel hepático durante el estado postprandial es suprimir la producción de glucosa mediante la inhibición de la glucogenólisis (degradación de glucógeno a glucosa) y la gluconeogénesis (biosíntesis de glucosa a partir de precursores no glucídicos). En pacientes con DMT2 se ha observado que defectos en las moléculas de señalización de insulina son un hallazgo común que se correlaciona con la hiperglucemia, tal y como se viene hablando a lo largo de este apartado. La RI en los hepatocitos da como resultado una síntesis alterada de glucógeno, mantenimiento de la gluconeogénesis e inducción de la DNL y de la síntesis de proteína C reactiva, retroalimentando el sistema y agravando la situación de RI (Meshkani & Adeli, 2009). El exceso de AGL provenientes de la lipolisis del tejido adiposo llegan al hígado a través de la circulación portal e inhibe la señalización de insulina. Este tejido también produce leptina y adiponectina, adipoquinas que se han clasificado como antidiabetogénicas por su capacidad para disminuir la síntesis de triglicéridos, estimular la β-oxidación y mejorar la acción de la insulina tanto en el músculo esquelético como en el hígado (Bazotte y cols., 2014). Curiosamente, los niveles de leptina se ven aumentados y los de adiponectina disminuidos en humanos y animales con RI, lo que sugiere que la DMT2 es un estado de resistencia a la leptina, que contribuye al el estado insulinorresistente y favorece el depósito de lípidos hepáticos (Muoio & Newgard, 2008). Dado que el hígado tiene una gran implicación en el metabolismo lipídico, el desarrollo de dislipemia como consecuencia de la RI suele ser otro factor que deteriora la salud hepática.

2.1.1. Estrés oxidativo como inductor de resistencia a la insulina

La evidencia actual señala al estrés oxidativo como un participante clave en el desarrollo de RI. El estrés oxidativo se origina por un desequilibrio entre las especies reactivas de oxígeno (ROS, de sus siglas en inglés *reactive oxygen species*) y los mecanismos antioxidantes presentes en el organismo, lo que puede conducir a la oxidación de enzimas, proteínas, lípidos y ácido desoxirribonucleico (ADN), generando toxicidad y daño celular (Betteridge, 2000). En condiciones fisiológicas, el sistema antioxidante endógeno mantiene un equilibrio redox dinámico. Sin embargo, se puede ver alterado por una producción excesiva de ROS y especies reactivas de nitrógeno (RNS, de sus siglas en inglés *reactive nitrogen species*) que conducen a un estado oxidativo dañino para el organismo, tal y como ocurre en diversas enfermedades crónicas como la DMT2 (Hurrle & Hsu, 2017). A pesar de que todavía no se ha

dilucidado completamente los mecanismos moleculares que relacionan estrés oxidativo y RI, sí que hay algunas pistas que sugieren esta conexión.

En situaciones de hiperglucemia las mitocondrias tienen más sustratos disponibles para producir ATP, lo que conduce a una hiperactividad por parte de este orgánulo y a una sobreproducción de ROS. El aumento del flujo glucolítico aumenta la fosforilación oxidativa y la generación de ATP, con la consiguiente liberación de anión superóxido por la enzima NADPH oxidasa de la cadena de transporte de electrones. La formación de superóxido se considera la primera etapa en una cascada generadora de ROS que produce otras formas de ROS/RNS (Yan, 2014). La hiperglucemia mantenida puede conducir a la generación de ROS a través de otros mecanismos, incluida la autooxidación de la glucosa y la generación de productos finales de glicosilación avanzada (AGE) que incrementan los niveles intracelulares de ROS. Este aumento de ROS/RNS daña la infraestructura celular e induce respuestas al estrés, estimulando algunas rutas proinflamatorias como NFkB, JNK, IKK y p38 MAPK (Hurrle & Hsu, 2017). La mayor disponibilidad de glucosa y AGL puede producir fisión mitocondrial como consecuencia del incremento de ROS celular, lo que aumenta la expresión de p38 MAPK y regula a la baja la función de IRS1 y AKT, alterando notablemente la señalización de insulina (Gao y cols., 2010). De la misma forma, estudios en adipocitos y miocitos han revelado que el peróxido de hidrógeno reduce la señalización de insulina por fosforilación de IRS1 en Ser^{307}, proceso que favorece la degradación de dicha proteína y dificulta la transducción de señales (Potashnik y cols., 2003). Un ejemplo similar lo encontramos en el hígado, donde intermediarios lipídicos como los diacilgliceroles o la ceramidas son capaces de incrementar el estrés oxidativo y la fisión mitocondrial, favoreciendo el desarrollo de RI y esteatosis (Roden & Shulman, 2019).

Es importante destacar que las células β pancreáticas presentan bajos niveles de enzimas antioxidantes, lo que las hace más vulnerables ante la hiperglucemia crónica característica de las personas con diabetes. Parece ser que niveles elevados de ROS activan la señalización de JNK, disminuyendo la secreción de insulina a través de la translocación núcleo-citoplasma de PDX-1, un factor de transcripción clave que se une al promotor de insulina e induce la expresión de dicha hormona. La activación por estrés oxidativo de las rutas de señalización mencionadas anteriormente (JNK, NFkB y p38 MAPK), son capaces de inducir apoptosis y provocar muerte celular en el páncreas (Newsholme y cols., 2019). Estos efectos agravarían la fisiopatología de la DMT2 al provocar la disfunción de la célula β pancreática.

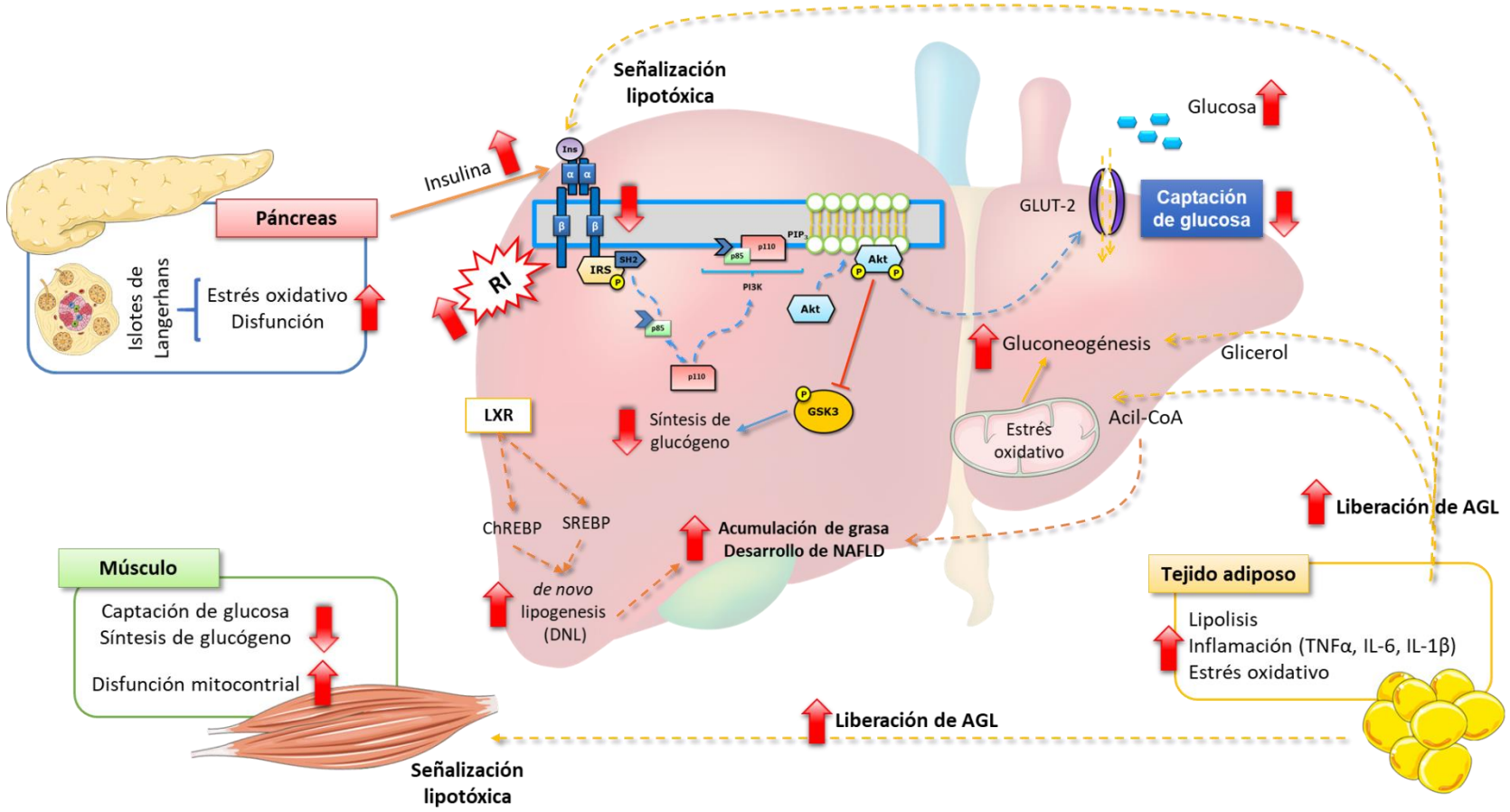

Figura 4. Esquema representativo de la fisiopatología de la DMT2

AGL, ácidos grasos libres; ChREBP, proteína de unión al elemento de respuesta a carbohidratos; GLUT-2, transportador de glucosa tipo 2; GSK3, glucógeno sintasa quinasa 3; Ins, insulina; IL-1β, interleuquina 1β; IL-6, interleuquina 6; LXR, receptor X hepático; NAFLD, enfermedad de hígado graso no alcohólico; PI3K, fosfatidilinositol 3-quinasa; RI, resistencia a la insulina; SREBP-1c, proteína de unión al elemento regulador del esterol; TNFα, factor de necrosis tumoral α.

2.2. Dislipemia diabética asociada a la DMT2

Al igual que la glucosa, los lípidos plasmáticos también se ven fuertemente alterados en la DMT2, asociándose en mayor medida con el riesgo de enfermedad cardiovascular (ECV). La dislipemia diabética es una alteración asociada a la RI que se caracteriza por anormalidades lipídicas cuantitativas, cualitativas y en su cinética, entre las que destacan la hipertrigliceridemia, niveles elevados de LDL pequeñas y densas, y una disminución de HDL (Hirano, 2018; Verges, 2015).

En la situación postprandial de individuos con DMT2 se ha observado un aumento en la producción de quilomicrones asociado a una mayor tasa de secreción intestinal de Apolipoproteína B-48 (ApoB-48) y una mayor expresión de la proteína responsable de la adición de triglicéridos a ApoB, la proteína microsomal transferidora de triglicéridos (MTP, de sus siglas en inglés *microsomal triglyceride transfer protein*) (Hogue y cols., 2007; Phillips y cols., 2006). Además, la eliminación de los quilomicrones también se ha visto alterada en esta patología, debido a una reducción de lipoproteína lipasa (LPL), enzima responsable de la hidrólisis del quilomicrón y las VLDL (Adiels y cols., 2012).

Continuando con el metabolismo lipoproteico, el aumento del flujo portal de AGL observado en sujetos con DMT2 estimula la síntesis de triglicéridos hepáticos, los cuales son empaquetados y devueltos a la circulación sistémica en forma de VLDL. En dicha patología, la función supresora de la insulina sobre la producción de VLDL está deteriorada como consecuencia de la RI, lo que conduce a una sobreproducción de VLDL grandes enriquecidas en triglicéridos. DNL se ve aumentada en sujetos con RI (Verges, 2015). Esta mayor formación de depósitos lipídicos es secundaria a la expresión aumentada de la proteína de unión al elemento de respuesta a carbohidratos (ChREBP, de sus siglas en inglés carbohydrate responsive element-binding protein) y de la proteína de unión al elemento regulador del esterol (SREBP-1c, de sus siglas en inglés *sterol regulatory element-binding protein*), factores que se asocian con el enriquecimiento en triglicéridos de las VLDL (Hirano, 2018). Unido a ello, se ha observado una disminución de su catabolismo, siendo los responsables en gran medida de la hipertrigliceridemia encontrada en este grupo de personas.

En la DMT2, la producción de LDL no es excesivamente elevada, pero la reducción en su catabolismo prolonga la vida media de este tipo de lipoproteínas, haciéndolas más susceptibles a la oxidación y favoreciendo su deposición en las paredes arteriales (Verges, 2005). Este menor aclaramiento de LDL es debido tanto a los menores niveles de receptor para LDL (LDLr) encontrados en personas con DMT2,

como a la glicosilación de las LDL como consecuencia de la hiperglucemia, lo cual disminuye su afinidad y dificulta su unión al receptor Apoe/ApoB100-LDL. Además de la menor tasa de eliminación, en la DMT2 las LDL se vuelven pequeñas y densas (sdLDL, de sus siglas en inglés *small dense-LDL*). Los niveles de estas sdLDL se correlacionan de forma directa con la cantidad de VLDL ricas en triglicéridos, ya que su formación está mediada por la proteína de transferencia de éster de colesterol, la cual intercambia los triglicéridos de las VLDL por el colesterol de las LDL. Posteriormente, la mayor afinidad de la lipasa hepática por las LDL ricas en triglicéridos acaba originando sdLDL al consumir los triglicéridos almacenados, las cuales son potencialmente más aterogénicas y presentan un mayor riesgo cardiovascular (Carmena, 2010; Sánchez-Quesada & Pérez, 2013; Verges, 2015).

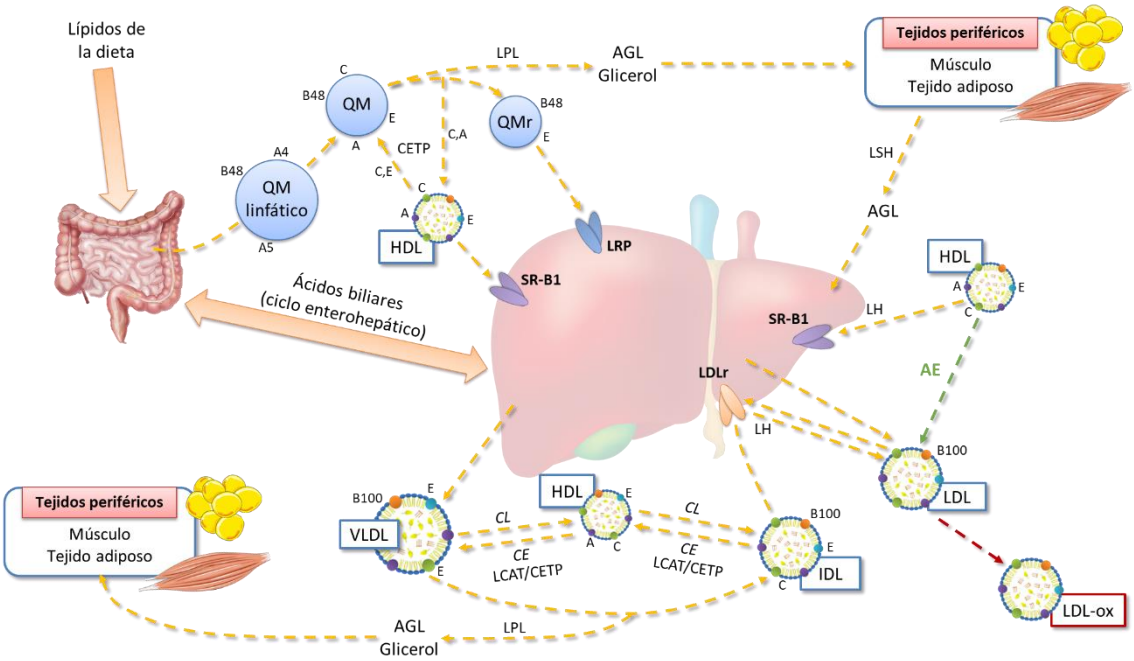

Figura 5. Rutas principales del metabolismo lipoproteico.

AE, arilesterasa; AGL, ácidos grasos libres; CE, colesterol esterificado; CETP, complejo de transferencia de ésteres de colesterol; CL, colesterol libre; HDL, lipoproteínas de alta densidad; IDL, lipoproteínas de densidad intermedia; LCAT, lecitin colesterol acil-transferasa; LDL, lipoproteínas de baja densidad; LDLox, LDL oxidadas; LDLr, receptor de LDL; LH, lipasa hepática; LPL, lipoprotein lipasa; LRP, proteína receptora de quilomicrones parecida a LDL; LSH, lipasa sensible a hormonas; QM, quilomicrones; QMr, quilomicrones remanentes; SR-B1, receptor *scavenger* tipo B1; VLDL, lipoproteínas de muy baja densidad. Modificado de Gesteiro (2015).

Las HDL son las encargadas de retirar el colesterol de los tejidos y transportarlo hacia el hígado para su eliminación, permitiendo establecer una asociación entre altos niveles de HDL y protección frente a ECV. Sin embargo, los valores plasmáticos de

HDL se ven reducidos en pacientes con DMT2. En concreto, la proporción de partículas HDL grandes, ricas en colesterol y conocidas como HDL_2, está disminuida; mientras que se observa una mayor presencia de HDL pequeñas (HDL_3) (Garvey y cols., 2003). A su vez, este tipo de lipoproteínas presenta anormalidades cualitativas, con mayor contenido en triglicéridos y glicosilación. Estudios cinéticos con radioisótopos e isótopos estables han demostrado que la disminución del colesterol HDL en pacientes con DMT2 se debe a un aumento de su catabolismo. La actividad de la lipasa hepática, enzima que controla el catabolismo de las HDL y que presenta mayor afinidad por lipoproteínas ricas en triglicéridos, aumenta en los estados resistentes a la insulina; siendo esta la principal consecuencia del aumento del catabolismo de las HDL (Despres y cols., 1989). Por otra parte, los productos finales de glicosilación avanzada inducidos por hiperglucemia, el estrés oxidativo y la inflamación de bajo grado son posibles mecanismos para promover la disfunción de las HDL (Meshkani & Adeli, 2009).

2.3. Enfermedad de hígado graso no alcohólico

El hígado graso no alcohólico, también conocido como NAFLD de sus siglas en inglés *non-alcoholic fatty liver disease*, se ha convertido en la enfermedad hepática más común en los países ricos, afectando hasta un tercio de los adultos en Europa y Estados Unidos. Anteriormente había sido considerado como la manifestación hepática del síndrome metabólico, pero en la actualidad la evidencia epidemiológica sugiere una relación bidireccional entre NAFLD y DMT2, donde el NAFLD puede preceder y/o promover el desarrollo de DMT2 (Mantovani y cols., 2018).

El NAFLD se define como la acumulación de grasa en forma de triglicéridos en más de un 5% de los hepatocitos en ausencia de inflamación o fibrosis y no asociada a un consumo elevado de alcohol (<30g en hombres y <20g en mujeres) (Abd El-Kader & El-Den Ashmawy, 2015; Chalasani y cols., 2012). Es una patología dinámica que puede avanzar desde esteatosis simple macrovesicular (NAFLD) hacia esteatohepatitis no alcohólica (NASH, por sus siglas en inglés *non-alcoholic steatohepatitis*), la cual se caracteriza por necroinflamación, apoptosis de los hepatocitos, fibrosis y cirrosis, con mayor predisposición para desarrollar carcinoma hepatocelular. El NAFLD se ve fuertemente influenciado por algunos factores de riesgo como la raza, sexo, edad, obesidad, hábitos de vida poco saludables, síndrome de ovario poliquístico o algunos componentes genéticos (ej. genes PNPLA3 y TM6SF2) (Kanwar & Kowdley, 2016). Su desarrollo aumenta el riesgo de mortalidad por

enfermedad hepática y cardiovascular, siendo a su vez la principal causa de carcinoma hepatocelular sin presencia de cirrosis (Yki-Jarvinen, 2016).

A pesar de que la patogenia del NAFLD es multifactorial y todavía no está bien caracterizada, todos señalan a la RI como uno de los mecanismos implicados en el desarrollo de esta patología. Por ello y dado que presentan fisiopatología común, se considera a la RI el nexo de unión entre el NAFLD y la DMT2. De acuerdo a esto, la hipótesis del "doble impacto" descrita por Day y James en 1998 es la que tiene mayor apoyo en la actualidad (Day & James, 1998). En primer golpe consiste en la acumulación de triglicéridos en los hepatocitos, fundamentalmente como consecuencia de la RI en el tejido adiposo blanco. Como se ha mencionado en los apartados anteriores, esta disfunción en el tejido adiposo induce lipolisis y la consiguiente liberación exacerbada de AGL al torrente sanguíneo, los cuales alcanzan el hígado provocando lipotoxicidad. La aparición de esteatosis se produce por una alteración en el balance entre el aporte de lípidos (exceso de grasas en la dieta, mayor captación de AGL circulantes y estimulación de la DNL a través de ChREBP y SREBP-1c), y el catabolismo de los mismos (β-oxidación de ácidos grasos y/o la secreción de triglicéridos en forma de VLDL) (Buzzetti y cols., 2016). El segundo impacto favorece la progresión de NAFLD a NASH, encontrando como factores favorecedores al estrés oxidativo, peroxidación lipídica, disfunción mitocondrial, apoptosis y respuesta inflamatoria de los hepatocitos (Kitade y cols., 2017).

2.4. Microbiota intestinal y DMT2

El término microbiota se define como el conjunto de microorganismos que reside en nuestro cuerpo, mientras que microbiota intestinal hace referencia exclusivamente a aquellos microorganismos localizados en el aparato digestivo. Todos ellos conforman el ecosistema más complejo, diverso y numeroso de nuestro cuerpo, especialmente localizado en el ciego, donde se encuentra la mayor densidad de microorganismos. Un adulto sano alberga 500–1000 especies bacterianas a la vez y puede haber 10^{12}–10^{14} unidades formadoras de colonias (UFC) en todo el intestino, con un peso total aproximado de 1–2 kg. El colon contiene alrededor de 10^{9}–10^{12} UFC/mL, seguido de 10^{1}–10^{3} UFC/mL en yeyuno y 10^{4}–10^{8} UFC/mL en el íleon (S. Sharma & Tripathi, 2019). Estudios metagenómicos han revelado que aproximadamente el 90% de las especies bacterianas presentes en el intestino de un individuo adulto pertenecen al filo *Bacteroidetes* (Gram-negativos) y *Firmicutes* (Gram-positivos), con menor proporción de *Proteobacteria*, *Verrucomicrobia*, *Fusobacteria*, *Cyanobacteria*, *Actinobacteria* y *Spirochaetes* (del Campo-Moreno y cols., 2018).

Entre las principales funciones de la microbiota intestinal están metabolizar los sustratos no digeribles (principalmente fibra dietética, aunque también pueden usar como sustrato proteínas o péptidos) en el tracto digestivo superior y producir ácidos grasos de cadena corta (AGCC), prevenir la colonización por otros microorganismos patógenos, estimular la producción de vitamina K y del grupo B, y modular el sistema inmune (Rowland y cols., 2018). Dada la gran implicación que muestra en la salud del individuo, cambios en la cantidad y diversidad de la microbiota intestinal, conocidos como disbiosis, se han asociado con la progresión de muchos trastornos metabólicos, entre los que se encuentra la obesidad, el síndrome metabólico y la DMT2 (Aw & Fukuda, 2018). La literatura actual que relaciona la microbiota con la DMT2 es un poco confusa, ya que los microorganismos que la conforman varían en función del individuo, la alimentación o el grado de desarrollo de la patología. Sin embargo, sí que parece que hay un patrón que se reproduce de manera común y es consistente entre personas con DMT2 (Tabla 1).

Tabla 1. Cambios en la microbiota asociados a la DMT2

Bacterias asociadas a la DMT2	
Asociación positiva	**Asociación negativa**
↑ *Firmicutes/Bacteroidetes* *Bifidobacterium* *Bacteroides* *Faecalibacterium* *Akkermansia* *Roseburia* *Lactobacillus?*	↓*Firmicutes/Bacteroidetes* *Ruminococcus* *Fusobacterium* *Blautia*

*Datos obtenidos y ordenados de acuerdo a la evidencia científica descrita hasta la fecha. (Gurung y cols., 2020)

A día de hoy, es muy difícil establecer cuál es el mecanismo desencadenante de la DMT2, siendo la interacción de muchos de ellos los que conforman su fisiopatología. La disbiosis de la microbiota intestinal parece ser un factor de gran relevancia en el desarrollo de DMT2. Esta alteración lleva asociados numerosos cambios que también han sido observado pacientes con IR, como una menor producción de AGCC, disfunción de la barrera mucosa intestinal, estrés oxidativo e inflamación (Gerard & Vidal, 2019; Muñoz-Garach y cols., 2016; Sharma & Tripathi, 2019). Todos estos procesos están interconectados y se mantienen en equilibrio en individuos sanos; sin embargo, en personas con DMT2 se pierde la homeostasis intestinal.

2.4.1. Producción de AGCC y su implicación en la DMT2

La microbiota intestinal es la encargada de producir AGCC, compuestos orgánicos que contienen de dos a seis átomos de carbono, al fermentar fibras dietéticas o aminoácidos no digeridos. El acetato (C2), propionato (C3) y butirato (C4) representan el 95% de los AGCC, pero también encontramos otros compuestos minoritarios derivados de la fermentación de los aminoácidos de cadena ramificada valina, isoleucina y leucina, que originan los AGCC isobutirato , 2-metilbutirato e isovalerato (Gerard & Vidal, 2019). Se producen en el colon y el ciego del huésped, pero su rápida absorción por el epitelio colónico permite una gran difusión por todo el organismo. El butirato se considera una fuente esencial de energía para los colonocitos, mientras que el acetato y el propionato se distribuyen por la circulación sistémica, siendo este último un sustrato para la gluconeogénesis (Puddu y cols., 2014). Los AGCC fortalecen la función de barrera epitelial al promover el crecimiento epitelial, la formación de moco y la respuesta innata frente a patógenos (Bilotta & Cong, 2019). No obstante, los niveles de AGCC se han visto reducidos en personas con DMT2 respecto a la población sana (Puddu y cols., 2014). Tanto la disbiosis propia de dicha patología (con reducción de especies productoras de butirato como *Firmicutes*, *Roseburia intestinalis* y *Faecalibacterium prausnitzii*), como unos hábitos alimentarios inadecuados con dietas con bajo contenido en fibra dietética, parecen ser los responsables de la baja producción de AGCC. La fibra es un sustrato esencial para la microbiota, cuyo consumo favorece el crecimiento de bacterias intestinales productoras de AGCC y reduce el riesgo de padecer DMT2 (Andoh y cols., 2003).

Tal y como se ha venido comentado en este apartado, los AGCC, además de ser una fuente importante de energía para los colonocitos, regulan el metabolismo de glucosa y lípidos mediante la activación de receptores para AGCC (FFAR, por sus siglas en inglés *free fatty acid receptor*; también denominados GPR, ya que son receptores acoplados a proteínas G) en el hígado, tejido adiposo, cerebro y páncreas (Utzschneider y cols., 2016). Tang y cols. revelaron en estudios en animales que los AGCC mantuvieron la homeostasis de la glucosa al interactuar con FFAR2/GPR43 y FFAR3/GPR41, lo que condujo a un aumento de la masa de células β pancreáticas y la secreción de insulina, a la vez que redujo la secreción de glucagón (Tang y cols., 2015). Otros estudios mostraron que la suplementación con propionato o butirato mejoró la homeostasis de la glucosa en roedores (Lin y cols., 2012). Asimismo, la administración de butirato disminuyó la esteatosis, inflamación y resistencia a la insulina hepática en un modelo animal con RI inducida por dieta alta en grasa (Raso y cols., 2013). La gran mayoría de los datos expuestos nos permiten ver el efecto

beneficioso de los AGCC sobre la RI, ya que también se ha observado que estimulan la oxidación de ácidos grasos e inhiben la DNL, protegiendo a su vez contra el desarrollo de NAFLD (Utzschneider y cols., 2016).

2.4.2. Modulación del metabolismo de la glucosa por la microbiota

La microbiota intestinal puede afectar la homeostasis de la glucosa y RI en los tejidos periféricos, así como regular en cierto modo la digestión de hidratos de carbono y la secreción de hormonas intestinales. Estas últimas, denominadas incretinas, son liberadas por las células enteroendocrinas para estimular la secreción de insulina y regular la hiperglucemia postprandial. Fundamentalmente están representadas por el péptido similar al glucagón tipo 1 (GLP-1, *glucagon-like peptide 1*), el polipéptido inhibidor gástrico (GIP, *gastric inhibitory polypeptide*) y el péptido YY (PYY). GLP-1 y PYY son las hormonas intestinales que mayor asociación han mostrado con algunos grupos bacterianos, en concreto con aquellos que incrementan la producción de AGCC o indol (Chimerel y cols., 2014). El acetato y el propionato son los activadores más potentes de GPR43, un receptor localizado en las células enteroendocrinas, cuya activación promueve la secreción de GLP-1 y ayuda a regular la glucemia (Tolhurst y cols., 2012). De la misma forma, se ha observado que los AGCC producidos por la microbiota (propionato y butirato) interaccionan con GPR41 en las células L intestinales e incrementan la secreción de PYY (Samuel y cols., 2008). Además algunas especies del género *Lactobacillus*, como *L. gasseri* o *L.casei*, han demostrado mejorar la RI al aumentar la expresión de PI3K, IRS2, AKT2, AMPK o GLUT en el hígado y músculo de modelos diabéticos (Kang y cols., 2013; Li y cols., 2017). *L. rhamnosus* aumenta el nivel de adiponectina en el tejido adiposo blanco, favoreciendo la sensibilización a la insulina en los tejidos periféricos (Singh y cols., 2017).

Como se puede observar en estos apartados, existe un importante vínculo entre la producción de AGCC por la microbiota, las hormonas enteroendocrinas, la sensibilidad a la insulina y la homeostasis de la glucosa (Tabla 2), lo que nos permite resaltar la importancia de modular la microbiota a través de una adecuada alimentación para mejorar la fisiopatología de la DMT2.

Tabla 2. AGCC e implicación sobre la salud.

AGCC	Productores	Receptor	Funciones derivadas de su unión al receptor	Función principal
Acetato (C2)	*Akkermansia muciniphila* *Bacteroides* spp. *Bifidobacterium* spp. *Prevotella* spp. *Ruminococcus* spp. *B. hydrogenotrophica* *Clostridium* spp. *Streptococcus* spp.	GPR43/FFAR2	Metabolismo: antilipolítico, aumento de la sensibilidad a la insulina y gasto energético, secreción de GLP-1 y PYY, diferenciación de preadipocitos y control del apetito Inmune: expansión y diferenciación de células Treg, aumento de células T contra bacterias patógenas, quimiotaxis de neutrófilos.	Circulación sistémica (efectos diversos en función del órgano diana)
Propionato (C3)	*Bacteroides* spp. *Phascolarctobacterium succinatutens* *Dialister* spp. *Veillonella* spp. *Megasphaera elsdenii* *Coprococcus catus* *Salmonella* spp. *Roseburia inulinivorans* *Ruminococcus obeum*	GPR43/FFAR2 GPR41/FFAR3	Metabolismo: antilipolítico, aumento de la sensibilidad a la insulina y gasto energético, secreción de GLP-1 y PYY, diferenciación de preadipocitos y control del apetito Inmune: expansión y diferenciación de células Treg, aumento de células T contra bacterias patógenas, quimiotaxis de neutrófilos. Metabolismo: aumento del gasto energético, tasa de consumo de oxígeno, expresión de leptina, disminución de la ingesta de alimentos, aumento de la expresión de PYY y gluconeogénesis intestinal. Inmune: hematopoyesis de células dendríticas de médula ósea, aumento de células Treg e inmunidad protectora.	Metabolismo hepático (Gluconeogénesis hepática e intestinal) Saciedad y disminución de la producción de glucosa hepática
Butirato (C4)	*Coprococcus comes* *Coprococcus eutactus* *Anaerostipes* spp. *Coprococcus catus* *Eubacterium rectale* *Eubacterium hallii* *F. prausnitzii* *Roseburia spp.*	GPR41/FFAR3 GPR109A/HCA2	Metabolismo: aumento del gasto energético, tasa de consumo de oxígeno, expresión de leptina, disminución de la ingesta de alimentos, aumento de la expresión de PYY y gluconeogénesis intestinal. Inmune: hematopoyesis de células dendríticas de médula ósea, aumento de células Treg e inmunidad protectora. Metabolismo: antilipolítico y disminución de triglicéridos Inmune: mejora de la barrera epitelial, aumento de la generación de células Treg, células T productoras de IL-10 y disminución de las células Th17 proinflamatorias	Fuente de energía para los colonocitos Aumento de la secreción de mucina Inhibición de histona desacetilasas

Modificada de Koh y cols. (2016). GLP-1, péptido similar al glucagón tipo 1; GPR, receptores acoplados a proteínas G; IL-10, interleuquina 10; PYY, péptido YY; Treg, células T reguladoras.

2.4.3. Microbiota intestinal, integridad de barrera e inflamación

La barrera gastrointestinal es una frontera fisiológica que limita el contacto entre el contenido luminal y las células intestinales, evitando el paso de bacterias comensales, patógenos y antígenos alimentarios que puedan llegar a la circulación sistémica. En el colon se compone de una barrera epitelial constituida en su mayoría por colonocitos, los cuales están fuertemente interconectados por uniones estrechas y proteínas de adhesión; y una capa mucosa que se relaciona de forma directa con la salud del huésped (Martin & Devkota, 2018). Ambas interaccionan de forma directa con la microbiota intestinal y sus metabolitos, proporcionando una homeostasis de barrera adecuada. Se ha propuesto la pérdida de la integridad de barrera como un impulsor de la inflamación crónica de bajo grado que se observa en la DMT2 (Cani y cols., 2007). La base bioquímica de la permeabilidad intestinal sigue siendo investigada, pero parece deberse a una interacción entre la dieta, la microbiota intestinal y sus metabolitos (Desai y cols., 2016).

En la DMT2 se producen en el colon tanto cambios funcionales como morfológicos que son indicativos de una deficiente integridad de barrera intestinal (Zhao y cols., 2017). Estas modificaciones podrían estar motivadas por la combinación de hiperglucemia y disbiosis, las cuales se ha relacionado con mayor permeabilidad intestinal, translocación de productos bacterianos a circulación sistémica y posible colonización de patógenos oportunistas (Martin & Devkota, 2018). Todo ello parece contribuir a la inflamación crónica de bajo grado que se asocia a esta patología, donde la producción excesiva de citoquinas como IL-6, IL-1, IL-8 o TNFα, dificultan la señalización de insulina, favorecen la RI y el desarrollo de alteraciones asociadas (Cani y cols., 2007). Shi y cols. ya demostraron que las bacterias intestinales pueden iniciar el estado inflamatorio característico de la DMT2 y la obesidad a través de la actividad del lipopolisacárido (LPS) o endotoxina, un componente de las paredes celulares de bacterias Gram-negativas que se une a TLR-4 (Toll-like receptor 4) de las células inmunes innatas y desencadena una respuesta proinflamatoria (Shi y cols., 2006). La microbiota intestinal se considera el principal reservorio de LPS en el organismo. En el caso de la DMT2, una dieta con alto contenido en grasas, la disbiosis, la descomposición de la integridad y el aumento de la permeabilidad intestinal favorecen la translocación de LPS desde la luz intestinal al torrente sanguíneo, causando lo que se conoce como endotoxemia metabólica (Gomes y cols., 2017). El LPS liberado por la microbiota intestinal puede activar TLR-4 en los islotes pancreáticos, aumentado la producción de citoquinas proinflamatorias y deteriorando

la función y viabilidad de las células β, retroalimentando el círculo vicioso de la DMT2 (Puddu y cols., 2014).

Anteriormente se ha hecho referencia a los AGCC como reguladores del metabolismo de los hidratos de carbono y las grasas, pero es importante destacar que estos compuestos orgánicos pueden actuar como inmunorreguladores al reducir la producción de citoquinas proinflamatorias tanto en las células inmunes como de forma local (Moffa y cols., 2019). La administración de butirato a animales alimentados con dieta rica en grasa, mejora la esteatosis e inflamación hepáticas, fundamentalmente debido a a la reducción de TNFα, IL-1β e IL-6 (Raso y cols., 2013). También pueden estimular la producción de células T reguladoras y equilibrar el status inflamatorio al suprimir su componente proinflamatorio (Arpaia y cols., 2013). Por otra parte, los AGCC regulan la integridad de la barrera intestinal al inducir la secreción de IL-18 por las células epiteliales intestinales, péptidos antimicrobianos, mucina, y regular al alza la expresión de las uniones estrechas (Sun y cols., 2017). La bibliografía actual describe efectos similares de los AGCC en los diferentes tejidos del organismo y células inmunes como consecuencia de la ubicuidad de sus receptores (FFAR/GPCR), aunque la gran mayoría de autores hacen hincapié en la capacidad de modular la función de leucocitos y adipocitos e inhibir la producción de citoquinas proinflamatorias (Puddu y cols., 2014; Roelofsen y cols., 2010).

Teniendo en cuenta toda esta información, podemos definir a la DMT2 como una patología multifactorial de la que todavía se tienen grandes dudas. En la actualidad se desconoce cuál es el factor desencadenante, por lo que de momento es necesario relacionar de forma conjunta todas las alteraciones que se en esta patología de forma conjunta.

3. Algarroba

El algarrobo (*Ceratonia siliqua L.*) es un árbol de hoja perenne de la familia *Fabaceae* que se encuentra en países de la región mediterránea, fundamentalmente en España, Italia, Marruecos, Grecia, Chipre, Turquía y Siria. Se pueden cultivar en áreas con poca lluvia ya que no requieren grandes cuidados, pudiendo alcanzar hasta los 150 años. Antiguamente, su fruto era muy utilizado tanto para la alimentación humana como animal, pero la mejora del nivel de vida determinó casi exclusivamente su utilización como forraje para la cría de ganado (Papaefstathiou y cols., 2018; Stavrou y cols., 2018).

La fruta del algarrobo recibe el nombre de algarroba y es la principal materia prima usada en la industria. Es una vaina coriácea, de color marrón y superficie arrugada, cuya estructura alargada es aproximadamente de 10-30 cm de largo, 1,5-3,5 cm de ancho y 1 cm de espeso. La vaina está compuesta de la pulpa, constituida por una parte más externa (pericarpio) y una región interna más suave (mesocarpio) que conforman el 90% del fruto; y las semillas que constituyen el 10% restante (Papaefstathiou y cols., 2018). De forma general, las algarrobas contienen un 48-56% de azúcar (principalmente sacarosa, fructosa y glucosa), 3-4% de proteína, 0,2-0,6% de grasa y un alto contenido de fibra dietética, pero su composición es diferente en relación a la parte del fruto a la que hagamos referencia (Ayaz y cols., 2007; Naghmouchi y cols., 2009; Papaefstathiou y cols., 2018). La pulpa de algarroba tiene un alto contenido en azúcar, fibra, minerales y polifenoles (con gran proporción de taninos condensados), mientras que las semillas son más ricas en grasas y pobres en azúcares en comparación con la pulpa (Stavrou y cols., 2018). Cabe mencionar que su composición varía en función de la región de cultivo y la etapa de crecimiento, encontrando una mayor variabilidad en humedad, azúcares, fibra dietética, proteínas, grasas y polifenoles (Figura 6) (Gharnit & Ennabili, 2016; Gubbuk y cols., 2010; Naghmouchi y cols., 2009).

Las semillas de algarroba se utilizan fundamentalmente para la producción de una harina denominada goma de algarroba o garrofín, cuyas propiedades espesantes, estabilizantes y aromatizantes confieren notables usos en la tecnología de los alimentos. Asimismo, también se usa en la industria farmacéutica como un agente portador para la liberación controlada de fármacos o por sus propiedades beneficiosas frente a las enfermedades gastrointestinales. Por otra parte, la vaina de algarroba ha despertado gran interés por su potencial nutricional, principalmente por ser una fuente muy rica de fibra dietética y compuestos bioactivos. Entre sus aplicaciones lo encontramos como sustituto del cacao, en diferentes productos de panadería y confitería, en alimentos funcionales como bebidas lácteas a base de algarroba o con aplicaciones biotecnológicas (Goulas y cols., 2016; Stavrou y cols., 2018; Zhu y cols., 2019). Actualmente, diversos estudios han demostrado los beneficios asociados al consumo de la pulpa de algarroba, especialmente por su elevada cantidad de fibra, ciclitoles y la gran diversidad de polifenoles que contiene, entre los que destacamos a los taninos condensados, también conocidos como proantocianidinas (PACs). Todos ellos se han relacionado con efectos antioxidantes, antidiabéticos, antidiarreicos, antihiperlipémicos y anticancerosos, fomentando su uso en diferentes patologías (Bastida y cols., 2009; El Hajaji y cols., 2011; Rtibi y cols., 2017).

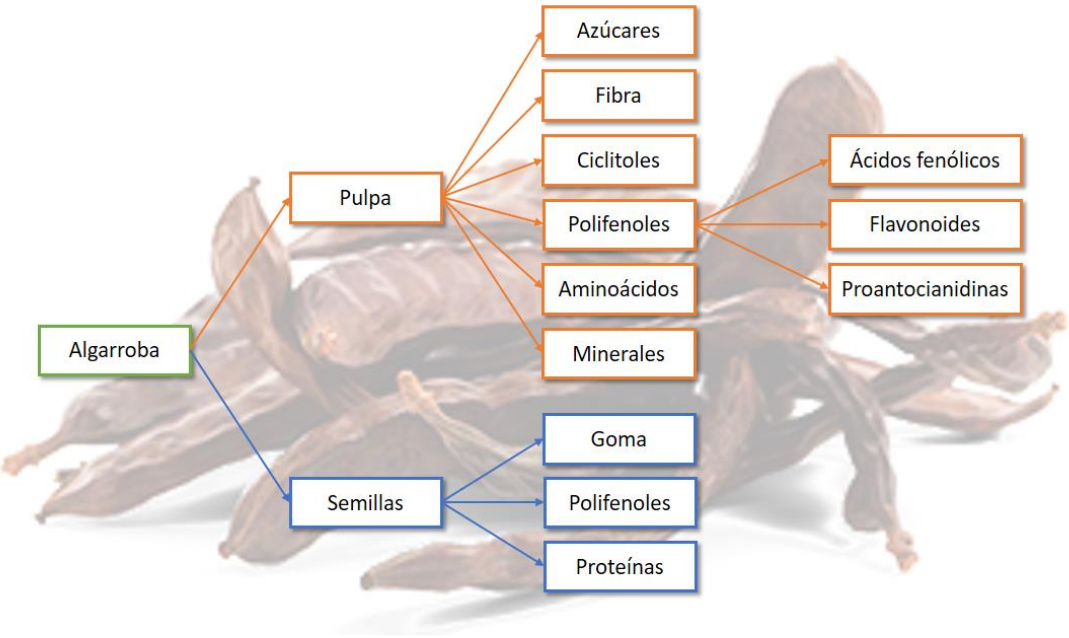

Figura 6. Principales componentes de la pulpa y semilla de la algarroba.

3.1. Fibra dietética como componente principal de la pulpa de algarroba.

La fibra dietética es un grupo heterogéneo de componentes alimenticios que son resistentes a la digestión y absorción en el intestino delgado pero que sufren una fermentación parcial o completa en el intestino grueso. Comúnmente se clasifica en fibra insoluble, escasamente fermentable y en cuya acepción se recoge a la celulosa, hemicelulosa de tipo B y lignina; y fibra soluble, viscosa y fermentable, donde se encuentran las hemicelulosas de tipo A, pectinas, gomas y mucílagos, entre otros (Sánchez-Muniz, 2012). La fibra predominante en la pulpa de algarroba es de tipo insoluble y no fermentable, aunque durante el proceso de extracción con agua, se arrastra una pequeña cantidad de fibra soluble e hidratos de carbono simples que no suponen más del 2% (Goulas y cols., 2016; Rtibi y cols., 2017). La producción de la fibra de algarrobo comienza con la eliminación de las semillas, que se procesan por separado para la obtención de la goma de algarrobo, obteniéndose una matriz viscosa compuesta de celulosa, hemicelulosa y lignina. Estos polisacáridos suelen ir unidos a diferentes componentes fenólicos a través de enlaces de hidrógeno entre los grupos hidroxilo de los polifenoles y los átomos de oxígeno de los enlaces glucosídicos de los polisacáridos, aunque también pueden presentar interacciones hidrofóbicas y enlaces covalentes (Figura 7). Esta asociación favorece la llegada de los polifenoles al colon, donde sufren un proceso de biotransformación por la microbiota intestinal para generar diferentes metabolitos activos que son transportados al torrente sanguíneo para su distribución (Zhu y cols., 2019).

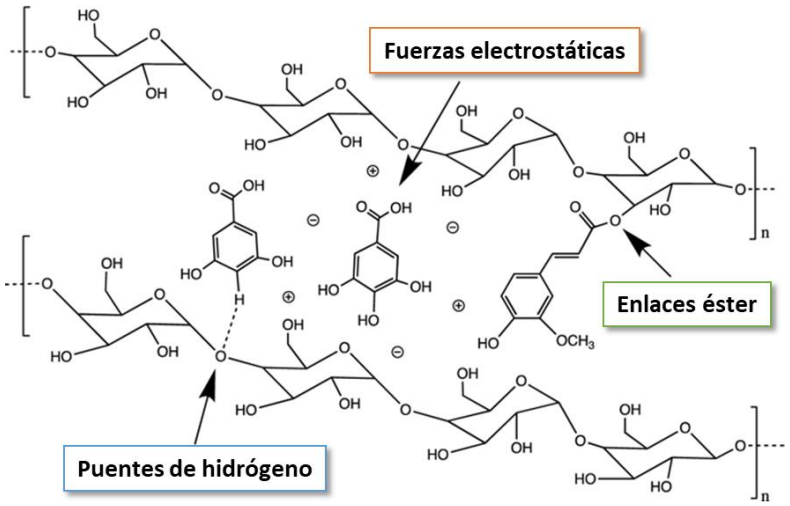

Figura 7. Tipos de interacciones entre polifenoles y fibra dietética

Imagen obtenida de Zhu y cols. (2019)

3.2. Polifenoles asociados a la pulpa de la vaina de algarroba.

Los polifenoles o compuestos fenólicos son un amplio grupo de compuestos orgánicos derivados del metabolismo secundario de las plantas y en cuya estructura contienen un anillo aromático con uno o más grupos hidroxilo. En el reino vegetal desempeñan numerosas funciones como defensa frente a patógenos, resistencia a enfermedades, regulación del crecimiento, etc.; además de ser responsables del sabor y color de las plantas (Stavrou y cols., 2018). Los compuestos polifenólicos se dividen generalmente en varias clases, de acuerdo con los anillos de fenol que contienen y las diferencias estructurales en la unión entre estos anillos. Los polifenoles se clasifican en subcategorías dependiendo de su estructura, encontrando compuestos más simples como los ácidos fenólicos o mucho más complejos como las PACs. Generalmente, su clasificación se centra en el número de átomos de carbono presentes en su estructura donde se encuentran los ácidos fenólicos, flavonoides, taninos (hidrolizables y condensados), estilbenos y lignanos (Spencer y cols., 2008).

La presencia de estos compuestos bioactivos dentro del fruto del algarrobo, al igual que en otras plantas, varía en función de los factores abióticos presentes en la zona de cultivo. Asimismo, es importante destacar que la composición polifenólica se ve modificada en función de si nos referimos a las semillas o a la pulpa (Papagiannopoulos y cols., 2004). Esta segunda fracción contiene una mayor cantidad y diversidad de polifenoles en comparación con las semillas, estableciéndose una media de entre 45-5376 mg de equivalentes de ácido gálico por 100g de extracto seco (Goulas y cols., 2016; Rico y cols., 2019). Tal y como se aprecia en la Tabla 3, los polifenoles mayoritarios presentes en la vaina de algarroba pertenecen al grupo de los

flavonoides. Su estructura química consta de un esqueleto difenilpropano (C_6-C_3-C_6) formado por dos anillos aromáticos (A y B) unidos a entre sí a través de tres átomos de carbono que conforman un heterociclo oxigenado (C). Los flavonoides se dividen en diferentes subcategorías en función del estado de oxidación del heterociclo, encontrándose flavanonas, flavonoles, flavonas, isoflavona, antocianidinas y flavanoles.

Tabla 3. Principales polifenoles encontrados en la algarroba

Parte del fruto	Clase de polifenoles	Polifenoles mayoritarios
Pulpa de algarroba	Benzaldehidos	Hidroxibenzaldehídos
	Ésteres galoilo de flavanol	(-)-Galato de epicatequina, (-)-galato de epigalocatequina
	Flavanoles	(-)-Epicatequina, (-)-epigalocatequina, (+)-catequina
	Flavonas	Hidroxiflavonas, apigenina, crisoeriol, luteolina, tricetina 3', 5' dimetiléter
	Glucósidos de flavona	Glucósido metoxi-genkwanina
	Flavanonas	Naringenin, eriodictyol
	Glucósidos de flavanona	Glucósido de naringenina
	Flavonoles	Kaempferol, miricetina, quercetina e isorhamnetin
	Glucósidos de flavonoles	Glucósidos de kaempferol, miricetina y quercetina
	Derivados de galatos	Galato de metilo
	Isoflavonas	Genisteína y derivados
	Glucósidos de isoflavona	Glucósido de genisteína
	Ácidos fenólicos	Ácido 3,4-dihidroxibenzoico, ácido 4-hidroxibenzoico, ácido cinámico, ácido cafeico, ácido cumárico, ácido elágico, ácido ferúlico, ácido gálico, ácido siringico, ácido vanilico
	Taninos	Derivados de galloil-β-D-glucosa, procianidinas, proantocianidinas, prodelfinidinas, digalloyl-glucosa y derivados, ácido tánico
Semillas	Flavanoles	(+)-Catequina
	Ácidos fenólicos	Ácido gálico y vanílico
	Taninos	Ácido tánico
	Trihidroxibenceno	Pirogalol

Tabla adaptada de Stavrou y cols. (2018).

3.2.1. Proantocianidinas o taninos condensados

Los flavanoles reciben su nombre por estar formados por unidades de flavan-3-ol, las cuales se unen dando lugar a estructuras poliméricas que conforman uno de los compuestos de mayor interés dentro de la pulpa de algarroba, las PACs. Este tipo de polifenoles se clasifican de acuerdo al número unidades de flavan-3-ol en su estructura (grado de polimerización), la posición de los grupos hidroxilo en los anillos aromáticos y la configuración espacial que origine la molécula (Rauf y cols., 2019). Teniendo en cuenta el grado de polimerización, en la naturaleza se pueden encontrar monómeros, dímeros, trímeros, tetrámeros o polímeros (grado de polimerización de 1, 2, 3, 4 ó >4, respectivamente) (Ou & Gu, 2014). Asimismo, según el patrón de hidroxilación se pueden observar PACs compuestas por unidades de catequina y epicatequina, denominadas en conjunto procianidinas; aunque en menor medida también encontramos propelargonidina (unidades de (epi) afzelequina) o prodelfinidina (unidades de (epi) galocatequina), entre otras (Ferreira & Slade, 2002). Por último, atendiendo a la clasificación estructural, las PACs se dividen en dos grandes grupos (A y B) en función del tipo de unión que presenten. En el grupo B, las unidades de flavan-3-ol se encuentran unidas por un enlace carbono-carbono entre las posiciones 4 y 6 (C4-C6) ó 4 y 8 (C4-C8) (Figura 8). Estas PACs del grupo B suelen estar constituidas en gran medida por unidades de procianidinas, siendo las más abundantes las de unión C4-C8. Las PACs de tipo A, además del enlace interflaváníco del tipo B, presentan una unión adicional entre el carbono C2 y el hidroxilo del carbono C7, conociéndose muy pocas fuentes naturales con este tipo de enlace.

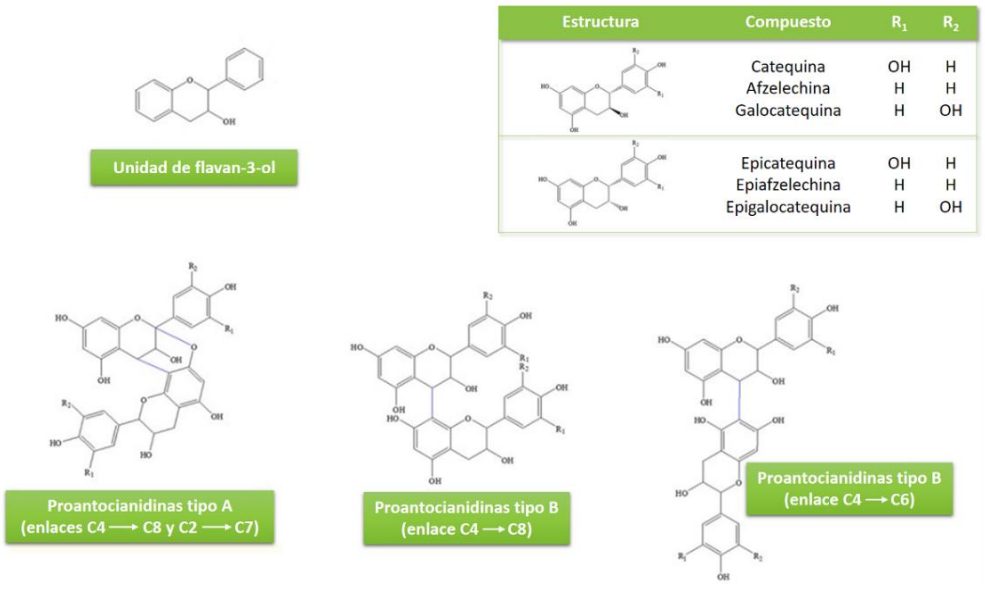

Figura 8. Estructura química básica de las PACs y sus conformaciones monoméricas

3.2.1.1. Biotransformación de las proantocianidinas

Las PACs son conocidas por su baja absorción intestinal como consecuencia de su naturaleza polimérica y su elevado peso molecular, pudiéndose establecer una relación inversa entre el grado de polimerización y la tasa de absorción (Ou & Gu, 2014). De forma general, en alimentos como los arándanos, uvas o algarrobo encontramos PACs con un grado de polimerización superior a 4, lo que condiciona notablemente su absorción y favorece su llegada al colon, donde son metabolizadas por la microbiota. Las diferencias encontradas en los estudios de biodisponibilidad de PACs *in vitro* e *in vivo*, sugieren un importante papel de la microbiota, por lo que describiremos de forma detallada el proceso de biotransformación que sufren las estructuras más pequeñas frente a los polímeros.

Metabolismo y absorción de monómeros, dímeros y oligómeros de PACs: como hemos señalado, la biodisponibilidad depende del grado de polimerización. Así, las PACs más pequeñas difunden de forma pasiva haciéndose bioaccesibles para los enterocitos del intestino delgado (Deprez y cols., 2001). Estudios en humanos y animales han demostrado que los monómeros de flavan-3-ol, fundamentalmente (+)-catequina y (-)-epicatequina, pueden absorberse en el intestino delgado (Rein y cols., 2000; Spencer y cols., 2001; Yang y cols., 1998). Sin embargo, los oligómeros de PACs han revelado grandes diferencias y tasas de absorción, encontrando autores como Spencer que describieron un proceso de despolimerización en mezclas de (-)-epicatequina en su paso por fluido gástrico simulado, facilitando su absorción (Spencer y cols., 2000); y otros como Ríos o Serra que afirmaron la estabilidad de las procianidinas en su paso por el estómago e intestino delgado (Rios y cols., 2002; Serra y cols., 2010). Actualmente se afirma que las discrepancias y diferencias entre la biodisponibilidad de este tipo de polifenoles se deben al modelo de estudio y el tipo de PACs utilizados, confirmándose que los oligómeros con un grado de polimerización inferior a 5 son absorbibles en el intestino delgado (Ou & Gu, 2014).

Fisiológicamente, las PACs que atraviesan el enterocito son tratadas como xenobióticos, sufriendo un primer proceso de glucuronidación por la uridina 5'-difosfo-glucuronosiltransferasa (UGT) en la parte luminal del retículo endoplásmico. Posteriormente, cuando estos compuestos son absorbidos y llegan al hígado, necesitan una sulfatación y metilación por parte de las sulfotransferasas citosólicas (SULT) y la catecol O-metiltransferasa (COMT) (Choy & Waterhouse, 2014). Estos compuestos al ser excretados por la bilis, pueden regresar por la circulación enterohepática al hígado haciéndose biodisponibles.

Metabolismo y absorción de polímeros de PACs: la gran mayoría de oligómeros y polímeros de PACs ingeridos, llegan al colon sin sufrir ningún tipo de transformación. Sin embargo, la microbiota parece jugar un papel esencial en la biotransformación de este flavonoide, ya que en la orina de ratas alimentadas con procianidinas se encontró un metabolito derivado de este tipo de polifenoles, el ácido 3-(3'-hidroxifenil)-propiónico (Griffiths, 1962). Años más tarde, este autor confirmó su hipótesis y reveló la importancia de la acción biotranformadora de la microbiota al observar diferentes metabolitos en la orina de ratas tratadas o no con antibióticos y alimentadas con (+)-catequina (Griffiths, 1964; Choy & Waterhouse, 2014). Este trabajo supuso un avance enorme en la investigación del catabolismo de las proantocianidinas por la microflora intestinal, así como el posible efecto prebiótico que éstas podrían ejercer, regulando las vías de señalización celular y protegiendo la integridad de la barrera intestinal.

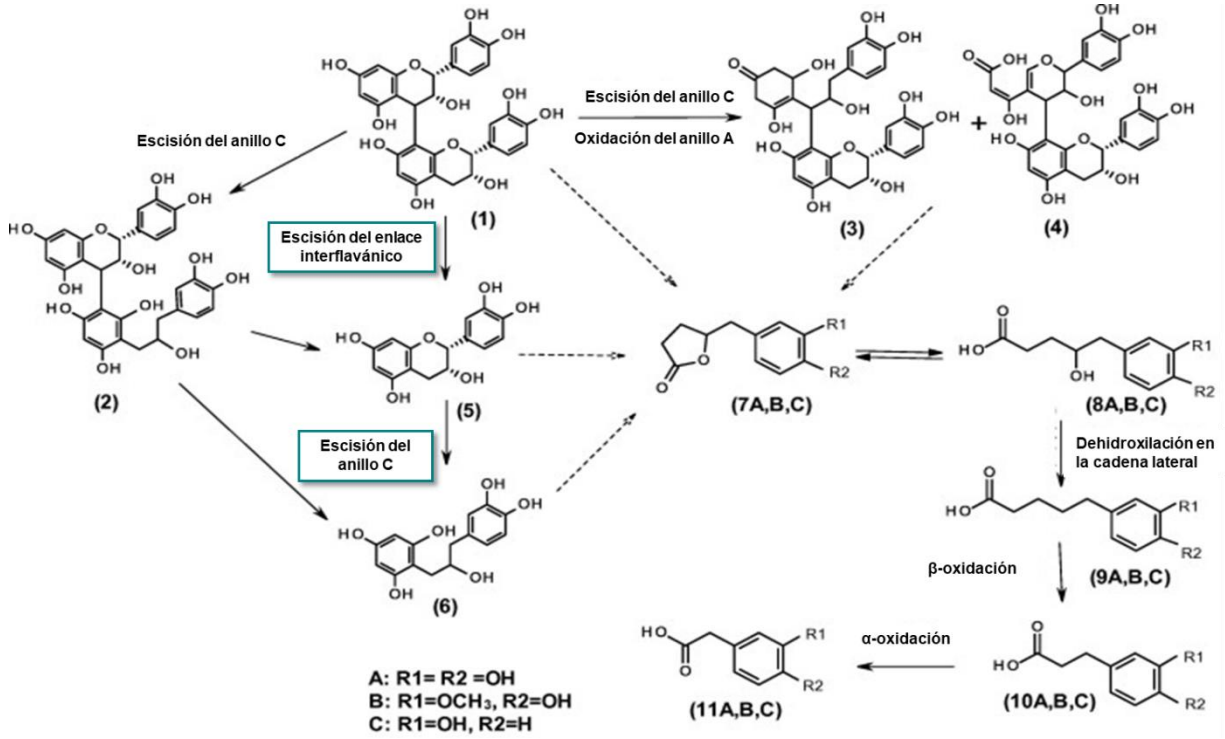

Figura 9. Catabolismo microbiano de un dímero de procianidina B2

Imagen modificada de Ou & Gu (2014).

Según Ou & Gu (2014) el proceso de biotransformación de las PACs varía en función de los grupos bacterianos presentes en el colon, así como el tipo de PACs ingerido. Estos autores utilizando un dímero de procianidina B2 (1) observaron que inicialmente las bacterias cortan el anillo C epicatequina en la posición C2 para formar (2) u oxidar el anillo A para formar los metabolitos (3) y (4). La microflora también puede escindir el enlace interflavan para convertir la procianidina B2 en dos (-)-epicatequinas (5). El dímero (1), sus productos de degradación inicial (2, 3, 4) y los

metabolitos monoméricos (5, 6) pueden ser a su vez degradados por la microflora intestinal, generando 5-(3', 4'-dihidroxifenil)-γ-valerolactona (7A), que puede ser metilada por catecol O-metiltransferasa para formar 5-(3 'methyoxyl, 4'-dihidroxifenil)-γ-valerolactona (7B). La microflora también puede eliminar un grupo hidroxilo de 7A para formar la 5-(3'-hidroxifenil)-γ-valerolactona (indicada en 7C). La deshidroxilación microbiana de los metabolitos se produce favorablemente en la posición C4 y en las cadenas laterales alifáticas, originando fenilvalerolactonas (8) que pueden degradarse lentamente en ácidos fenilvaléricos (9) tras la deshidroxilación de la cadena lateral. El acortamiento progresivo de la cadena alifática por oxidaciones α y β genera ácido fenilpropiónico (10), ácido fenilacético (11) y derivados del ácido benzoico (Figura 9) (Ou & Gu, 2014; Ou y cols., 2014).

De forma general, los compuestos mayoritarios procedentes del catabolismo de las PACs a nivel colónico son los ácidos fenólicos simples como los ácidos hidroxibenzoico, hidroxifenilacético, hidroxifenilpropinoico, hidroxifenilvalerico, hidroxicinámico, y sus formas dihidroxiladas. Se ha propuesto que *Clostridium* y *Eubacterium* son los principales géneros bacterianos involucrados en la metabolización de las PACs (Cires y cols., 2016). Finalmente, los metabolitos generados pueden ser absorbidos o conjugados en el hígado antes de ser eliminados por la orina (Choy & Waterhouse, 2014; Ou & Gu, 2014; Rauf y cols., 2019).

3.3. Beneficios para la salud derivados del consumo de algarroba

Numerosos estudios han atribuido propiedades beneficiosas al consumo de algarroba y sus derivados, tanto en estado de salud como en algunas patologías crónicas (Goulas y cols., 2016; Rtibi y cols., 2017; Zhu y cols., 2019). En este apartado describiremos los principales efectos beneficiosos atribuidos en la literatura a este vegetal y/o a sus extractos, tanto a nivel local, derivados de su paso a lo largo del tracto gastrointestinal; como a nivel periférico, asociados a los metabolitos activos producidos por fermentación colónica. Tal y como se ha comentado anteriormente, algunos polifenoles como las PACs atraviesan sin modificación el tracto gastrointestinal superior, y alcanzan el colon, donde son sustrato para la microbiota intestinal. En este proceso de biotransformación, se generan metabolitos que se distribuyen por todo el organismo y ejercen diferentes funciones, de las cuales aún no se conoce su mecanismo de acción. Las funciones beneficiosas más relevantes asociadas al consumo de PACs se esquematizan en la figura 10.

3.3.1. Efecto antioxidante de la algarroba y sus productos derivados

Las células son capaces de regular la respuesta al estrés oxidativo en función de la cantidad de ROS, activando genes que responden a los cambios del estado redox (Schieber & Chandel, 2014). Asimismo, diversos componentes de los alimentos como los polifenoles protegen a la célula frente a este desequilibrio al reducir la carga de ROS y modificar las vías de señalización implicadas en la maquinaria antioxidante, destacando la vía de activación del factor de trascripción Nrf2 que promueve la expresión de enzimas antioxidantes El potencial antioxidante de la algarroba y sus productos varía en función de la zona de origen, variedad, estado de maduración y la parte del fruto seleccionada, los cuales condicionan, a su vez, su composición polifenólica (Custodio y cols., 2011). El consumo de algarroba y sus productos derivados reducen, entre otros aspectos, la producción de ROS y la alteración del sistema de glutation hepático en ratas obesas (Fernández-Iglesias y cols., 2014), mejora la disfunción mitocondrial del tejido adiposo marrón asociada a una dieta obesogénica (Pajuelo y cols., 2012), y contrarresta la lipoperoxidación gástrica inducida por etanol en ratas Wistar (Rtibi y cols., 2015). Por otra parte, se ha comprobado en estudios *in vitro* con extractos de algarroba las propiedades antioxidantes mediante la determinación de 2,2-difenil-1-picrilhidrazilo (DPPH), formación de peroxidación lipídica a través de sustancias reactivas al ácido tiobarbitúrico (TBARS) o malondialdehído (MDA) (Bastida y cols., 2009; Roseiro y cols., 2013; Sebai y cols., 2013). Por otro lado, y dada la riqueza en PACs de la vaina de algarroba, cada vez es mayor su importancia económica y en el campo de la salud atendiendo a su efecto antioxidante. Especialmente se han señalado sus efectos tanto a nivel local en la mucosa colónica, como en los diferentes tejidos del organismo tras su biotransformación y transporte, tal y como recogieron (Scalbert y cols., 2002).

3.3.2. Efecto anticancerígeno de la algarroba y sus productos derivados

Como hemos detallado anteriormente (Tabla 3), la algarroba es rica en compuestos fitoquímicos, muchos de los cuales han demostrado tener actividad antitumoral, antiproliferativa y proapoptótica (Goulas y cols., 2016). La gran mayoría de estudios que describen estos efectos se basan en modelos *in vitro* realizados con diferentes líneas celulares. Los compuestos más estudiados han sido catequina, ácido gálico y sus combinaciones, demostrando sus propiedades antiproliferativas al inhibir la síntesis de ADN y ser inductoras de apoptosis en líneas celulares de hepatocarcinoma y adenocarcinoma colorrectal (Corsi y cols., 2002; Klenovv y cols., 2008; Klenow & Glei, 2009).

3.3.3. Efecto antidiarreico de la algarroba y sus productos derivados

La diarrea aguda se define como la deposición de heces líquidas 3 o más veces en un periodo de 24 horas (Nemeth y cols., 2019). Se ha observado que la algarroba, cuya composición se basa en azúcares, fibra y PACs, es una adecuada alternativa nutricional para el tratamiento de la diarrea (Theophilou y cols., 2017). En primer lugar la fibra insoluble de la algarroba ha demostrado efectos muy positivos sobre la diarrea en lactantes de entre 3 a 21 meses de edad (Loeb y cols., 1989). Es conocida el papel de la fibra dietética, fundamentalmente insoluble regulando la excreción fecal, en virtud de garantizar un volumen fecal adecuado (Sánchez-Muniz, 2012). De la misma forma, la rehidratación oral combinada con jugo de algarroba redujo en un 45% la presencia de diarrea en niños hospitalizados por dicha sintomatología (Aksit y cols., 1998). En segundo lugar, las PACs son ampliamente conocidas por sus propiedades astringentes y antimicrobianas. Su consumo se ha asociado a una menor adherencia de *Escherichia coli* en las células epiteliales intestinales, lo que justificaría la efectividad de los estudios anteriormente mencionados (Guggenbichler, 1983).

3.3.4. Efecto antidiabético de la algarroba y sus productos derivados

El control de la glucemia, especialmente durante la hiperglucemia postprandial, supone una de los retos más importantes para aquellos pacientes con DMT2. Es por ello, que como terapia combinada para el control de la glucosa plasmática se ha incrementado el consumo de numerosos compuestos de origen vegetal (Bindu & Narendhirakannan, 2018; Solayman y cols., 2016). Entre ellos se encuentra la algarroba y sus productos derivados, cuyo consumo es capaz de mejorar sensiblemente el metabolismo de los hidratos de carbono (Rtibi y cols., 2017) y de mostrar propiedades hipoglucemiantes tanto en modelos animales como en el hombre. Su elevada concentración de fibra dietética reduce y retrasa la absorción de glucosa en el tracto gastrointestinal, minimizando los riesgos de la hiperglucemia postprandial (Zhu y cols., 2019). Asimismo, en el caso de la pulpa o vaina de algarroba, la gran presencia de PACs podría reducir la digestión de los hidratos de carbono al inhibir las enzimas α-amilasa y α-glucosidasa, limitando la absorción de glucosa (Custodio y cols., 2015). Estos efectos pueden deberse, entre otros aspectos, a la presencia de D-pinito, un ciclitol presente en la algarroba, que ha mostrado propiedades insulinomiméticas, posiblemente activando la ruta InsR/IRS/PI3K/AKT (Gao y cols., 2015; Lambert y cols., 2018). A pesar de que existen algunos estudios que señalan el potencial antidiabético de la algarroba, la evidencia actual sigue siendo limitada y se necesita un mayor número de estudios que confirme estos efectos. La figura 10

resume algunos de los mecanismos implicados en la reducción de la digestión y metabolismo de los hidratos de carbono por los productos derivados da la algarroba.

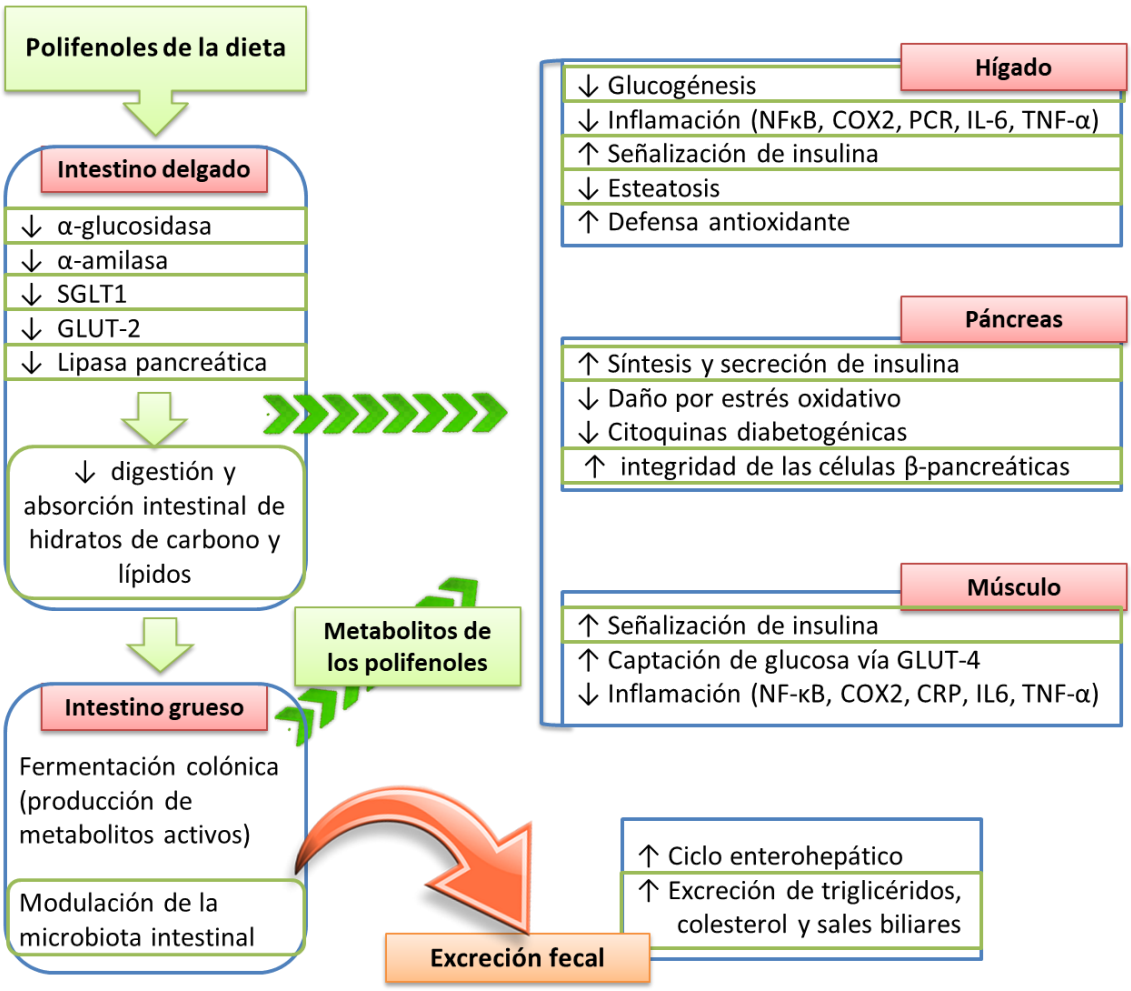

Figura 10. Efectos beneficiosos del consumo de PACs en el organismo.

Los recuadros verdes son indicativos de los efectos descritos para las PACs de la algarroba, incluyendo los resultados obtenidos en la presente Tesis Doctoral. ↑, incremento/mejora; ↓ disminución; COX2, ciclooxigenasa 2; GLUT-2, transportador de glucosa tipo 2; GLUT-4, transportador de glucosa tipo 4; IL-6, interleuquina 6; NFκB, factor nuclear kappa B; PCR, proteína C reactiva; SGLT1, cotransportador de sodio-glucosa tipo 1; TNFα, factor de necrosis tumoral α.

3.3.5. Efecto hipolipemiante de la algarroba y sus productos derivados

La propiedad hipolipemiante de la fibra dietética es ampliamente conocida, permitiendo establecer una asociación inversa entre su consumo y el desarrollo de ECV (Sánchez-Muniz, 2012). Según este mismo autor, aunque el mecanismo no está completamente definido, la fibra dietética atraparía en su malla cantidades variables de sales biliares, disminuyendo el retorno de estos componentes al hígado por el ciclo entero-hepático. La menor disponibilidad de ácidos biliares, implicaría, a su vez, la

activación enzimática hepática de la enzima colesterol 7 alfa-hidroxilasa, con la transformación de colesterol en ácido cólico y la consiguiente disminución del “pool” de colesterol libre hepático. Este efecto implicaría, por un lado, menores niveles de colesterol libre para ser empaquetados en las VLDL y, por otro, un mecanismo eficaz de regulación de las lipoproteínas aterogénicas, a través de regular la expresión de los receptores para apoE/B100. Según Brown & Goldstein (1997) la disminución del “pool de colesterol libre”, se regularía a través de la expresión del factor de transcripción SREBP. A su vez, según Dietschy (1998), la reducción del “pool” de colesterol libre induce una mayor transcripción de mRNA para los receptores de lipoproteínas aterogénicas conteniendo apoE/apoB100. Ruiz-Roso y cols. evaluaron el efecto hipocolesterolemiante del consumo de fibra insoluble de algarroba en pacientes hipercolesterolémicos. Los resultados reflejaron una marcada reducción del colesterol total, LDL y triglicéridos plasmáticos tras 4 semanas consumiendo 8 gramos de extracto de algarroba (Ruiz-Roso y cols., 2010). Otros autores también encontraron resultados similares con diferentes fracciones de la algarroba (Zavoral y cols., 1983; Zunft y cols., 2003). El efecto hipolipemiante parece ser consecuencia de una serie de factores: aumento en la excreción de ácidos biliares, lo que produciría una mayor demanda de su precursor, el colesterol; reducción de la absorción de lípidos, con su consiguiente eliminación fecal por el efecto arrastre de la fibra dietética; inhibición de la síntesis endógena por la acción de los AGCC generados en el colon; o por un aumento del metabolismo lipoproteico (Ruiz-Roso y cols., 2010).

4. La dieta como tratamiento para las enfermedades crónicas

Hace apenas 100 años, los agentes infecciosos eran la principal causa de enfermedad. A día de hoy, los estudios epidemiológicos han demostrado que la dieta juega un papel esencial en el riesgo de desarrollo de enfermedades crónicas, demostrándose que los patrones alimentarios inadecuados ejercen un marcado efecto sobre la salud (Schulze y cols., 2018; Shivappa, 2019). Los cambios en los hábitos alimentarios que caracterizan la “transición nutricional” se basan fundamentalmente en dietas desbalanceadas con mayor densidad energética, en las que prevalecen las grasas, especialmente saturadas, y azúcares simples; en detrimento de frutas y verduras, que aportan hidratos de carbono complejos y grasas. A su vez, el reducido gasto calórico como consecuencia de la inactividad física, contribuye a un balance energético positivo que conduce al desarrollo de enfermedades crónicas (Sánchez-Muniz & Sánz-Pérez, 2016). Esto va en contra de lo que ya predicaba Hipócrates en el siglo V a. C., quien definía salud como un equilibrio entre lo que nutre (los alimentos) y

lo que se gasta (el ejercicio). Este tipo de alimentación aumenta el riesgo de padecer ECV, obesidad, síndrome metabólico y DMT2, lo que supone un grave problema de salud pública a nivel mundial (Bellou y cols., 2018; IDF, 2019). Las actividades de prevención dirigidas a reducir este tipo de patologías se fundamentan en una dieta y estilo de vida saludables, tal y como recoge Sánchez-Muniz en las recomendaciones para reducir el riesgo de sufrir ECV (Sánchez-Muniz y cols., 2013).

4.1. Intervención nutricional en la DMT2

El tratamiento dietético de la DM ha sido descrito desde la antigüedad, encontrándose las primeras referencias en el papiro de Ebers, el cual data de 1550 a.C (Khazrai y cols., 2014). En la actualidad, aunque la modificación del estilo de vida y la dietoterapia se consideran las piedras angulares de la prevención y el tratamiento de la DMT2, no existe un consenso definitivo sobre qué tratamiento dietético es el más apropiado para prevenir el desarrollo de la patología, controlar la hiperglucemia e inducir una pérdida de peso duradera (Neuenschwander y cols., 2019).

La terapia nutricional se puede enfocar a dos grandes objetivos. El primero de ellos, dirigido a prolongar al máximo el tiempo desde que se detecta un metabolismo anormal de la glucosa (prediabetes) hasta que se instaura la enfermedad; y el segundo, centrado en prevenir y controlar las complicaciones de la DM (Khazrai y cols., 2014). Una vez que la persona ya tiene DMT2, la dietoterapia se centra en este segundo objetivo, buscando reducir el peso corporal y mantener niveles de glucosa y lípidos lo más normales posibles, ya que se ha observado que la reducción del peso corporal mejora notablemente la sensibilidad a la insulina (American Diabetes Association, 2020; Muoio & Newgard, 2008). Varios metaanálisis que describen el papel de la dieta sobre la incidencia de DMT2 asocian de forma inversa alimentos concretos (granos integrales, yogur, café, té y grasas vegetales) y el desarrollo de esta patología (Neuenschwander y cols., 2019). Sin embargo, los efectos derivados de un solo alimento no tienen suficiente poder estadístico para poder establecer una relación clara entre consumo y reducción del riesgo. Esta asociación entre dieta y riesgo es mucho más evidente en el caso de las dietas completas, según se deduce de algunas revisiones o metanálisis, situando a la dieta Mediterránea, la DASH (*Dietary Approaches to Stop Hypertension*) y la AHEI (*Alternative Healthy Eating Index*) entre las que mayor potencial han mostrado para prevenir la DMT2 (Jannasch y cols., 2017). La Tabla 4 muestra los posibles efectos beneficiosos de seguir un patrón nutricional concreto sobre los diferentes objetivos a abordar por parte de los profesionales de la nutrición ante un paciente con DMT2.

Es importante remarcar que no existe una dieta o un patrón nutricional de referencia ("*Gold standard*") para todos los pacientes con DMT2. La intervención nutricional debe hacerse de forma individualizada, ajustándose, dentro de lo posible, a las características y preferencias de cada persona y grado de desarrollo de la enfermedad. La ADA establece una serie de puntos clave a cumplir durante la dietoterapia (American Diabetes Association, 2020; Evert y cols., 2019):

- Promover y apoyar patrones de alimentación saludable, haciendo hincapié en una variedad de alimentos con gran densidad de nutrientes, con el objetivo de mejorar la salud general y específicamente para:
    - Mejorar los niveles de HbA1C, presión arterial y colesterol (ajustar el objetivo en función de edad, duración de la diabetes, historia clínica y condiciones específicas de cada individuo).
    - Lograr y mantener objetivos de peso corporal (reducción del 5-7%).
    - Retrasar o prevenir complicaciones de la diabetes.
    - Promover la actividad física (al menos 150 minutos/semana ≈ consumo de 700 kcal/semana).
- Abordar las necesidades nutricionales individuales de acuerdo a las preferencias personales y culturales (mantener el placer de comer), el acceso a opciones de alimentos saludables y la capacidad de realizar cambios de comportamiento. Intentar mantener el placer de comer
- Proporcionar herramientas prácticas para la planificación diaria de las comidas.
- Apoyo en la farmacoterapia en caso de ser necesario.

También se ha definido como aspecto clave que el porcentaje de energía aportado por los hidratos de carbono junto con los ácidos grasos monoinsaturados (AGM) sea entre el 60 y el 70%, y que el consumo de fibra dietética sea de >30 g/d (20g/1000 kcal), asegurando un aporte de cinco o más raciones al día de frutas más verduras, más de cuatro raciones/semana de legumbres y que los cereales procedan de grano completo (Connor y cols., 2003).

Tabla 4. Patrones nutricionales con mayor evidencia sobre la prevención y control de la DMT2

PATRÓN NUTRICIONAL	GRUPOS DE ALIMENTOS	BENEFICIOS DESCRITOS
De estilo mediterráneo	Alimentos vegetales (verduras, legumbres, nueces y semillas, frutas y granos integrales), pescado y otros mariscos, aceite de oliva como fuente principal de grasas, bajo/moderado consumo de productos lácteos (principalmente yogurt y queso), huevos, ingesta reducida de carne roja y vino en cantidades bajas/moderadas.	Menor riesgo de diabetes. Reducción de HbA1c Disminución de los triglicéridos. Reducción del riesgo de ECV.
DGA	Variedad de verduras, frutas (preferiblemente enteras), granos (50% integrales), lácteos bajos en grasa, variedad de alimentos proteicos y aceites. Este patrón de alimentación limita las grasas saturadas y *trans*, azúcares añadidos y sodio.	Reducción de HbA1c Reducción de LDL-C Reducción del riesgo de ECV.
Vegetariana o vegana	Los dos enfoques más comunes que se encuentran en la literatura enfatizan la alimentación: ovolactovegetariana (ingesta de vegetales, huevos y lácteos) y vegana (desprovista de todos los alimentos de origen animal).	Menor riesgo de diabetes. Reducción de HbA1c Pérdida de peso Reducción de LDL-C
Bajo en grasa	Verduras, frutas, almidones (p. ej. Panes, galletas saladas, pastas y granos integrales), alimentos ricos en proteínas y bajos en grasa, y productos lácteos bajos en grasa. En esta revisión, la dieta baja en grasa se establece con un aporte ≤30% de las kcal totales procedente de la grasa, donde las grasas saturadas comprendan ≤10%.	Reducción del riesgo de diabetes. Pérdida de peso
Muy bajo en grasa	Verduras ricas en fibra, legumbres, frutas, granos integrales, lácteos sin grasa, pescado y claras de huevo. Perfil calórico: 70–77% hidratos de carbono (30–60 g/día de fibra), 10% de grasa, 13–20% de proteína.	Pérdida de peso Disminución de la presión arterial.
Bajo en hidratos de carbono	Verduras con pocos hidratos de carbono (p. ej. brócoli, coliflor, pepino y repollo) y grasa de alimentos de origen animal, aceites, mantequilla y aguacate; proteínas de todas las fuentes animales. Evitar alimentos conteniendo almidón y azúcar como pasta, arroz, patatas, pan, dulces. En esta revisión, la dieta baja en hidratos de carbono se establece con un aporte del 26-45% de las kcal totales procedentes de los hidratos de carbono	Reducción de HbA1c Pérdida de peso Disminución de la presión arterial. Aumento de HDL-C y disminución de triglicéridos

Muy bajo en hidratos de carbono	Similar al patrón bajo en hidratos de carbono, pero con mayor limitación de los alimentos ricos en este macronutriente. En esta revisión, los hidratos de carbono aportan <26% de las kcal totales.	Reducción de HbA1c Pérdida de peso Disminución de la presión arterial. Aumento de HDL-C y disminución de triglicéridos
DASH	Verduras, frutas y productos lácteos bajos en grasa, granos integrales, nueces, aves de corral, y pescado. Reducción de sal, grasas saturadas, carnes rojas, dulces y bebidas azucaradas.	Reducción del riesgo Pérdida de peso Disminución de la presión arterial.
Paleo	Hace referencia a los alimentos teóricamente consumidos durante la evolución humana como la carne magra, pescado, mariscos, vegetales, huevos, nueces y bayas. Evita los granos, lácteos, sal, grasas *trans* y azúcares refinados.	Resultados mixtos Evidencia no concluyente

*Tabla adaptada de Evert y cols., (2019). DASH: Dietary Approaches to Stop Hypertension, DGA: Dietary Guidelines for Americans, ECV: enfermedad cardiovascular, HbA1C: hemoglobina glicosilada; HDL-C: colesterol transportado por las lipoproteínas de alta densidad; LDL-C: colesterol transportado por las lipoproteínas de baja densidad

4.2. Importancia de la carne en la dieta

Los patrones globales de hábitos alimentarios revelan que tanto el consumo promedio per cápita de carne como la cantidad total consumida están aumentando notablemente, siendo más elevado en los países industrializados, que permite establecer una relación positiva entre el nivel de ingresos y el consumo de proteína animal (Godfray y cols., 2018; Olmedilla-Alonso y cols., 2013). Utilizando datos promedio del consumo de carne a nivel mundial, se estima que cada ser humano consume alrededor de 122g/día, de los cuales un tercio es carne de cerdo y aves de corral, un quinto carne de res y el resto procede de otras fuentes, como carne de oveja o cabra (FAO, 2016). Este incremento viene dado en su mayoría por el mayor consumo que se ha observado en zonas del mundo con ingresos medios, especialmente en China y Asia Oriental; ya que simultáneamente parece existir una estabilización e incluso una ligera disminución del consumo en los países con altos ingresos (Godfray y cols., 2018).

En España, el pollo fue el tipo de carne más consumido en 2018, alcanzando aproximadamente 12,6 kg/persona/año, seguido del cerdo y la ternera, que contribuyeron con 9,99 y 4,9kg/persona/año, respectivamente. Como se puede observar, estos datos revelan la importancia que tiene este grupo de alimentos en la alimentación de nuestro país (Celada y cols., 2016). Concretamente, si valoramos la contribución de la carne y los productos cárnicos al perfil calórico de la dieta española media, se observa que el 4,8% y el 9,8% de las calorías de la dieta provienen de ellos. De la misma forma si lo evaluamos en relación a los objetivos nutricionales para la población española, que señalan que la dieta debe aportar un 10-15% de las kcal totales en forma de proteínas y un máximo del 35% de la kcal totales como grasa, la carne y derivados cárnicos aportan el 32,1-48,1% de las proteínas y el 28% de las grasas (Celada & Sánchez-Muniz, 2016; Ortega y cols., 2014). También señalar la contribución de este grupo de alimentos en micronutrientes, contribuyendo con el 16,7% de zinc, 13,3-24% de hierro y 17,1-13,4% de selenio a las ingestas dietéticas de referencia para la población española (mujeres y hombres, respectivamente) (Moreiras y cols., 2003).

La carne está constituida mayoritariamente por agua (60-80%), proteínas (16-25%) (aproximadamente el 40% de sus aminoácidos son esenciales) y grasas (1-30%). Su contenido en hidratos de carbono es casi inexistente, debido que el glucógeno de los músculos desaparece casi por completo durante el proceso de maduración. En la carne se encuentran pequeñas cantidades, sustancias nitrogenadas

no proteicas (aminoácidos libres, péptidos, creatina, nucleótidos, etc.), ácido láctico, vitaminas (tiamina, niacina, retinol y vitaminas B_6 y B_{12}), pequeña cantidad de vitamina D y minerales (hierro hemo y zinc de alta biodisponibilidad, así como fósforo, selenio, sodio, potasio y cobalto) (Chizzolini y cols., 1999; Olmedilla-Alonso y cols., 2013; Sánchez-Muniz, 2005). Aunque se habla de forma genérica, la composición de la carne varía en función del tipo de alimentación del animal, edad, tipo de producto, etc.; tal y como se detalla en la Tabla 5, donde se expone una comparativa de la composición de carne de vacuno y cerdo teniendo en cuenta su origen.

Tabla 5. Comparativa de la composición nutricional por 100g de carne magra de vacuno y cerdo procedente de España y EE.UU.

	VACUNO		CERDO	
	España	**EEUU**	**España**	**EEUU**
Energía (kcal)	131	126	155	144
Proteína (g)	20,7	21	20	21,2
Grasa (g)	5,4	4	8,3	5,9
AGS* (g)	2,2	1,4	3,2	2
AGM* (g)	2,5	1,6	3,6	2,7
AGP* (g)	0,2	0,2	0,6	0,6
Tiamina/B1 (mg)	0,1	0,1	0,9	1
Niacina/B3 (mg)	8,1	6,2	8,7	4,8
Vitamina B12 (µg)	2	1,5	3	0,7
Hierro (mg)	2,7	1,8	1,5	0,9
Cinc (mg)	3,8	3,9	2,5	2
Selenio (mg)	3	26	14	32,4
Sodio (mg)	61	54	76	54
Potasio (mg)	350	323	370	384

*AGS, ácidos grasos saturados; AGM, ácidos grasos monoinsaturados; AGP, ácidos grasos poliinsaturados. Adaptado de Celada & Sánchez-Muniz (2016).

4.3. Alimentos funcionales

La idea de desarrollar alimentos funcionales surgió por primera vez en Japón en la década de 1980 en un intento de mejorar la salud a través de la nutrición. El Ministerio de Salud y Bienestar inició un sistema regulatorio para aprobar ciertos alimentos con beneficios para la salud documentados, cuyo objetivo principal se basó en mejorar la salud del envejecimiento de la población del país (Henry, 2010). Esta organización estableció el término FOSHU (de sus siglas en inglés, *Foods for Specific Health Use,* en español, Alimentos de Uso Específico para la Salud) en el año 1991 (Sánchez-

Muniz, 2004) tomando relevancia internacional en 1993 cuando apareció en la revista *Nature* bajo el encabezado "Japón explora el límite entre alimentos y medicamentos" (Swinbanks & O'Brien, 1993). En Europa, este tipo de alimentos tuvieron su punto de partida en 1999 con la realización del proyecto FUFOSE (por sus siglas en inglés, *Functional Food Science in Europe*) por la Sección Europea del International Life Sciences (ILSI) y patrocinado por la Acción concertada de la Comisión Europea. El objetivo de este proyecto fue establecer un enfoque científico para el campo de los alimentos funcionales, así como establecer una red europea multidisciplinaria para: evaluar críticamente la base científica de los productos, examinar las evidencias disponibles desde una perspectiva basada en funciones en lugar de basada en productos y llegar a un consenso sobre las modificaciones específicas de los alimentos y sus componentes (Diplock y cols., 1999). De acuerdo al proyecto FUFOSE, los alimentos funcionales, también denominados FOSHU, son aquellos alimentos que demuestran científicamente un efecto beneficioso sobre una o más funciones del organismo, además de sus efectos nutritivos intrínsecos. Se recogen dentro de la categoría de nuevos alimentos y deben haber sido evaluados científicamente, de forma que se certifiquen las propiedades de mejora del estado de salud, reducción del riesgo de enfermedad, o ambas cosas (Diplock y cols., 1999).

Para que un alimento quede enmarcado dentro de esta categoría, es necesario que el producto alimenticio final sea el responsable del efecto beneficioso, no sus componentes individuales aislados, y que dicho efecto se obtenga con un consumo normal de ese alimento dentro de una dieta equilibrada (Ashwell, 2002). Para ello, los grupos encargados de desarrollar alimentos funcionales deben identificar y validar marcadores biológicos relevantes que sean susceptibles de modificación tras el consumo de los alimentos. Estos marcadores biológicos son diversos para el mundo de la alimentación funcional, pero de forma genérica se reconocen 3 tipos (Ashwell, 2002; Olmedilla-Alonso y cols., 2013):

a) Marcador de exposición: aquellos cambios que se producen en el organismo y que demuestran la exposición al componente del alimento en estudio (p. ej. determinación de los niveles en plasma de un metabolito concreto). Estos marcadores dan cierta información sobre la biodisponibilidad del componente funcional.
b) Marcador de respuesta/mejora de la función: capaces de modular o modificar alguna función biológica o psicológica tras su consumo (p. ej. cambios en los fluidos biológicos o el tránsito intestinal).

c) Marcador intermedio/reducción del riesgo de enfermedad: hacen referencia a un objetivo intermedio que refleje una mejora en el estado de salud o bienestar o una reducción del riesgo de enfermedad (p. ej. medida de los niveles de hemoglobina en relación con la anemia).

La obtención de un alimento funcional se puede conseguir a través de diferentes estrategias, en función del objetivo que se busque a la hora de su diseño. Las principales quedan recogidas en la figura 11.

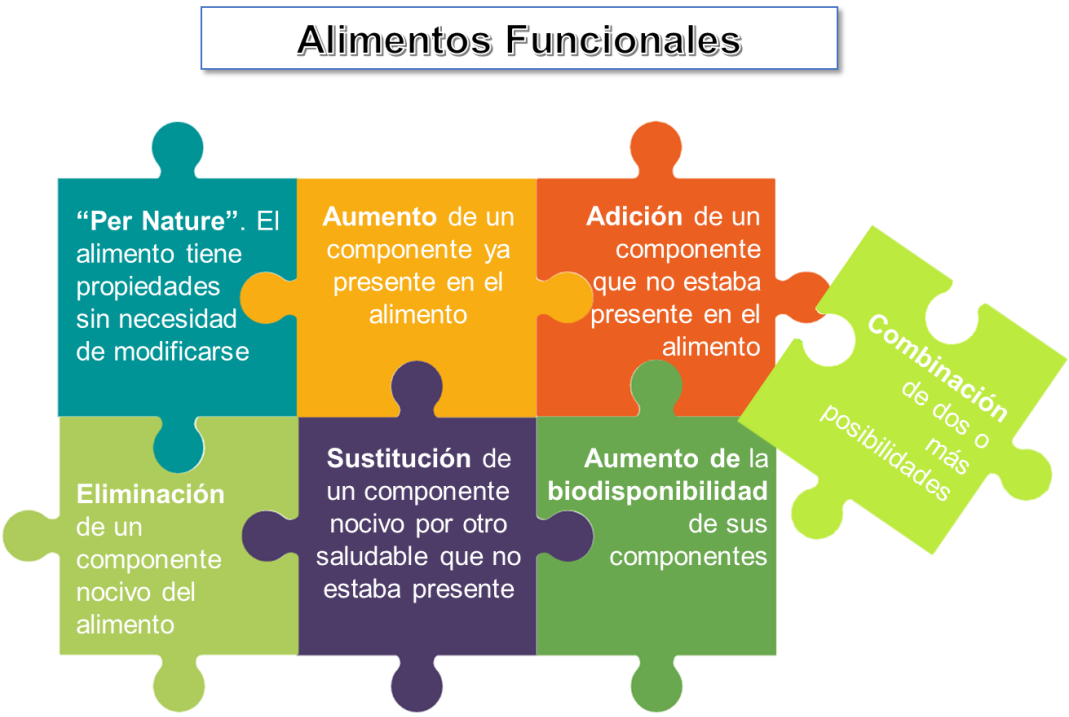

Figura 11. Principales estrategias a la hora de diseñar un alimento funcional.

En la actualidad ha aparecido el término nutracéutico, el cual se puede definir como un suplemento o complemento nutricional, presentado en una matriz no alimenticia (píldoras, cápsulas, polvo, etc.), de una sustancia natural bioactiva concentrada, presente usualmente en los alimentos y que, tomada en dosis superior a la existente de forma natural, muestra un mayor efecto beneficioso sobre la salud que el propio alimento (Gul y cols., 2016; Kalra, 2003). Teniendo en cuenta las definiciones de nutracéutico y alimento funcional las diferencias son evidentes, pero es importante destacar que el primero de ellos no tiene forma o estructura de alimento, sino que hace referencia a un compuesto aislado y se comercializa como forma farmacéutica, mientras que el alimento funcional en ningún momento pierde las propiedades del alimento que lo constituye.

4.4. Productos cárnicos como alimentos funcionales

La carne y los productos cárnicos, además de ser una excelente fuente de nutrientes, también presenta ciertos componentes que no son tan beneficiosos como los ácidos grasos saturados (AGS) y el colesterol, cuyo consumo en grandes cantidades, puede producir efectos adversos para la salud (Celada y cols., 2016). De hecho, varias revisiones recientes han restablecido una relación entre el consumo de carne roja y procesada y un mayor riesgo de ciertas enfermedades crónicas, principalmente ECV, cáncer y DMT2 (Aune y cols., 2009; Richi y cols., 2015). Sin embargo, algunas de estas propiedades negativas que se le atribuyen, son potencialmente mejorables aplicando modificaciones cuantitativas y cualitativas que se pueden resumir en tres grandes bloques. El primero, basado en las prácticas de producción animal, representa la primera oportunidad para modificar la presencia de componentes bioactivos, entre las que la incorporación de compuestos como aceites vegetales, minerales o vitaminas supone una estrategia adecuada para incrementar su concentración en el cárnico futuro. El segundo, se centra en estrategias genéticas para producir cambios favorables en los alimentos, la mayoría de ellas dirigidas a reducir el contenido de grasa total o incrementar el contenido de ácidos grasos insaturados. La tercera estrategia y más utilizada, es la reformulación de productos cárnicos con el objetivo eliminar, reducir, aumentar, agregar y/o reemplazar diferentes componentes bioactivos (Decker & Park, 2010; Jiménez-Colmenero y cols., 2001; Olmedilla-Alonso y cols., 2013) (Figura 11). Por tanto, dado que la carne tiene una amplia aceptación y es una matriz adecuada para vehiculizar componentes bioactivos, predispone a este alimento como una magnífica opción para el desarrollo de alimentos funcionales. Para su desarrollo, es necesario poder cumplir dos aspectos esenciales: la selección y caracterización de componentes bioactivos en el alimento, el diseño tecnológico, el desarrollo del alimento y la evaluación de factores que modifican su contenido/biodisponibilidad; y por otra parte, la evaluación del efecto funcional utilizando modelos *in vitro* e *in vivo* que proporcionen información sobre los mecanismos de acción, relación dosis-respuesta y efectos agudos y crónicos (Aggett y cols., 2005).

Nuestro grupo de investigación ha venido utilizando en mayor medida la tercera estrategia citada, con el objetivo de diseñar alimentos funcionales que demuestren sus posibles efectos beneficiosos sobre la salud tanto en humanos como en modelos animales. Algunos ejemplos de los principales ingredientes funcionales utilizados hasta la fecha han sido nueces, algas, glucomanano, espirulina, aceite de chía, silicio o hidroxitirosol, entre otros (Bocanegra y cols., 2009; Canales y cols., 2007;

Garcimartín y cols., 2017; González-Torres y cols., 2016; Librelotto y cols., 2008; Sánchez-Muniz y cols., 2012; Santos-López y cols., 2016; Schultz-Moreira y cols., 2010a). En el presente trabajo, el turno es para un extracto de algarroba, rico en fibra insoluble y PACs.

4.5. Extracto de algarroba como ingrediente funcional

Los alimentos cárnicos, por definición, son ricos en grasas y proteínas, pero son deficientes en otros compuestos como hidratos de carbono, fibra y ciertas vitaminas. Por tanto, enriquecer los productos cárnicos con ingredientes de origen vegetal supone un gran avance nutricional, ya que permite complementar muchos de los macro y micronutrientes deficitarios de la carne. Un ejemplo lo encontramos en la adición de fibra, cuya incorporación puede aportar excelentes propiedades tecnológicas (retención de agua, lubricación, capacidad para disminuir la pérdida de cocción, estabilización del producto, modificación de la textura y sabor neutro) y nutricionales (mayor aporte de fibra, mantenimiento de la microbiota intestinal, efecto hipocolesterolemiante, saciante o antioxidante) (Mehta y cols., 2015; Verma & Banerjee, 2010).

Los productos derivados del algarrobo cada vez están siendo más utilizados en la industria alimentaria como consecuencia de sus prometedoras aplicaciones tecnológicas. La goma de algarroba, la cual se obtiene de la semilla, se utiliza para elaborar recubrimientos comestibles que protejan los alimentos, como espesante para bebidas o en productos horneados como panecillos, pasteles y galletas, mejorando sus propiedades reológicas (Zhu y cols., 2019). Asimismo, miembros de nuestro equipo de investigación evaluaron la efectividad antioxidante del extracto de algarroba como ingrediente funcional de un reestructurado cárnico tras someterlo a almacenamiento refrigerado y congelado. Los resultados reflejaron una potente protección del extracto de algarroba al reducir la peroxidación lipídica y la formación de compuestos polares durante el almacenamiento (Bastida y cols., 2009). Como se ha comentado, las PACs tienen gran cantidad de grupos hidroxilo en su estructura, los cuales podría actuar como donantes de hidrógenos para estabilizar los procesos oxidativos y ejercer el efecto protector observado.

Teniendo en cuenta a estos prometedores efectos, queda por comprobar si tecnológicamente es posible desarrollar un cárnico funcional que contenga una cantidad adecuada de extracto de algarroba. (Freire y cols., 2018) elaboraron emulsiones gelificadas con ácidos grasos omega-3 como sustitutos de la grasa animal

y PACs añadidas. Ese estudio demostró que mediante este sistema es posible incorporar una cantidad más que notable de PACs en un cárnico reestructurado, mejorar su estabilidad y retrasar los procesos oxidativos de los ácidos grasos poliinsaturados (AGP) que contienen (Freire y cols., 2018). Por tanto, si tomamos en conjunto estas propiedades y la definición de alimento funcional, observamos que se cumplen las características necesarias para el desarrollo, en este caso, de un cárnico funcional enriquecido en extracto de algarrobo. Tecnológicamente es viable, permite incorporar una cantidad importante de extracto y mantiene el potencial antioxidante en su interior, por lo que nos quedaría por evaluar el efecto funcional derivado de su consumo en un modelo *in vivo*. En este caso, se han seleccionado dos modelos murinos de DMT2 que simulan una etapa temprana y una etapa tardía de la patología, tal y como se verá en la siguiente sección.

II. HIPÓTESIS Y OBJETIVOS

2.1. Interés del estudio

La dieta y el estilo de vida juegan un papel central en la prevención y tratamiento de algunas enfermedades crónicas como la DMT2. La dificultad de modificar los hábitos alimentarios en la población, unido a la evidencia creciente del papel saludable de muchos compuestos bioactivos procedentes de fuentes naturales, ha potenciado el diseño, elaboración, estudio y comercialización de los alimentos funcionales. Estos alimentos, acorde a su definición, aportan beneficios sobre la salud y el riesgo de patología crónicas más allá de los meramente nutricionales. Su consumo podría ser primordial para prevenir o ralentizar el progreso de ciertas patologías, especialmente en aquellos pacientes en los que el tratamiento farmacológico no alcanza la eficacia deseada.

La DMT2 es conocida como una patología silenciosa, prácticamente asintomática hasta que se instaura la enfermedad. Este periodo de tiempo supone un gran problema a la hora de diagnosticar la enfermedad, pero también permite establecer una terapia nutricional preventiva, que ayude a frenar o ralentizar el desarrollo de la o incluso una dietoterapia que minimice los efectos negativos de la RI previamente establecida. Como consecuencia, parece muy importante desarrollar estrategias nutricionales que permitan incrementar el aporte de ciertos compuestos bioactivos beneficiosos, como fibra y compuestos vegetales, sin necesidad de modificar en gran medida los hábitos alimentarios, que hoy por hoy constituye la piedra angular de la dietoterapia. La fibra dietética y algunos polifenoles han mostrado una asociación inversa con el desarrollo de DMT2, ya que minimizan las modificaciones glucémicas postprandiales y permiten regular el metabolismo de los hidratos de carbono y las grasas.

Nuestro grupo de investigación, al igual que otros que formaron el Consorcio Carnisenusa, lleva años profundizando en el estudio de los alimentos funcionales, utilizando la carne y los productos cárnicos como vehículo para incorporar compuestos bioactivos que mejoren el estado de salud del consumidor. A pesar de que se han realizado numerosos estudios epidemiológicos y en animales de experimentación que exponen los beneficios para la salud del consumo de alimentos funcionales, el conocimiento de cómo los compuestos bioactivos actúan sobre el organismo en ocasiones no está bien documentado.

2.2. Hipótesis y objetivos

El consumo de cárnicos funcionales enriquecidos en extracto de algarroba (CFE) rico en proantocianidinas (PACs) y fibra insoluble ejerce efectos beneficiosos, tanto preventivos como terapéuticos, sobre la fisiopatología de la Diabetes Mellitus Tipo 2 (DMT2) al influir positivamente sobre biomarcadores antropométricos y metabólicos, de sensibilidad/resistencia a la insulina, histopatología pancreática y hepática y de integridad colónica, así como sobre la disbiosis intestinal.

Teniendo en cuenta esta hipótesis, el **objetivo principal** de esta memoria de Tesis Doctoral es evaluar la eficacia del CFE como ingrediente funcional para formular productos cárnicos con propiedades antidiabéticas, así como caracterizar sus efectos sobre marcadores de la DMT2 y los principales mecanismos responsables de los mismos.

Para ello se propusieron los siguientes **objetivos específicos**:

1. Evaluar la capacidad del CFE para modular el estado postprandial a través de la inhibición de la digestión y absorción de hidratos de carbono y grasas, así como caracterizar los mecanismos moleculares implicados
2. Reflexionar a partir de la revisión exhaustiva de la literatura disponible la relación entre el consumo de carne y productos cárnicos y el desarrollo de patologías crónicas; y justificar el diseño de cárnicos funcionales enriquecidos con compuestos bioactivos como alternativa saludable.
3. Estudiar la viabilidad, ventajas e inconvenientes de la inclusión de un extracto de algarroba como ingrediente funcional en una matriz cárnica, con especial aplicación bajo el punto de vista preventivo y terapéutico de la DMT2.
4. Diseñar dos modelos murinos de DMT2 que repliquen las alteraciones fisiopatológicas de dos etapas diferentes (inicial y tardía) de la patología.
5. Evaluar las propiedades antidiabéticas del consumo de un cárnico funcional enriquecido con extracto de algarroba en las etapas inicial y avanzada de la DMT2, centrándose en su efecto efectos hipolipemiante, hipoglucemiante, antioxidante, hepatoprotector y prebiótico.
6. Evaluar comparativamente la eficacia del consumo preventivo o terapéutico de un cárnico funcional enriquecido en CFE en el marco de una DMT2 de estadio tardío.

Estos dos últimos objetivos se abordan a través de otros objetivos más concretos que se plantean con la finalidad de demostrar en animales tratados respecto a controles:

a. La mejora del estatus glucémico, insulinémico, disfunción pancreática y de resistencia a la insulina.
b. Las modificaciones en el balance glucogénesis/glucogenosis hepático y su implicación en el desarrollo y evolución del hígado graso no alcohólico.
c. Los beneficios sobre la lipemia y el estatus lipoproteico.
d. Los efectos sobre la abundancia y diversidad de la microbiota intestinal, producción de AGCC e integridad intestinal.

III. MATERIAL Y MÉTODOS

Para facilitar la lectura y el seguimiento de la presente Tesis Doctoral, en este apartado se van a describir tanto aspectos generales de los reactivos empleados como los principales métodos analíticos empleados, subdividiéndolos en técnicas *in vitro* e *in vivo*. Además, en la parte final de la descripción del método se detalla en qué artículo o artículos de la sección 4 se han empleado.

3.1. Proceso de obtención del Extracto de Algarroba

Para la obtención de los extractos ricos en PACs se empleó el fruto del algarrobo (*Ceratonia siliqua*, L), de la familia de las Fabaceae, reino Plantae, clase Magnoliopsida, orden Fabales. Las vainas fueron suministradas por la empresa Comercial Garrofa, Comgar, SCCL (Mont-roig del Camp, Tarragona, España).

La obtención del extracto de algarroba (CFE) se realizó de acuerdo a las patentes WO2004/014150 y WO2006/000551 (Ruiz-Roso y cols., 2006; Ruiz-Roso y cols., 2004) mediante el siguiente proceso.

- Limpieza de la vaina de la algarroba. Separar las semillas de la pulpa de algarroba y triturar esta última fracción hasta obtener trozos de menos de 3cm (1kg de material de partida).
- Realizar un lavado de la pulpa de algarroba triturada con agua a 60ºC, en una relación 1:4-1:10 del material vegetal frente al agua. Alternativamente se pueden realizar los lavados con agua:metanol (20:80, v/v) o agua:acetona (30:70, v/v), ambos a temperatura ambiente.
- Separar el sólido y el líquido resultante mediante centrifugación, filtración o decantación. Repetir esta operación tantas veces como sea necesario hasta obtener menos 50g de material por litro de líquido de lavado.
- Realizar una extracción del sólido obtenido en la etapa anterior a presión ambiente o presurizada con agua a temperatura entre 80-100ºC y agitación. Este proceso dura entre 3-4h y es importante mantener una relación de material vegetal:agua entre 1:2-1:3 (peso/peso). También se puede realizar este proceso de extracción utilizando otros disolventes:
  - Metanol: temperatura entre 40ºC-punto de ebullición y relación 1:4-1:6 (peso/peso) material vegetal:disolvente.
  - Mezcla agua:propilenglicol: temperatura entre 80ºC-punto de ebullición.
  - Dimetilformamida: temperatura entre 80ºC-punto de ebullición.
  - Dimetilsulfóxido: temperatura entre 80-150ºC.

- Separar el sólido y el líquido resultante mediante centrifugación, filtración o decantación, manteniendo una temperatura superior a 40ºC.
- Enfriar el líquido obtenido en el paso previo en tanques de decantación durante 4-10h. Durante el proceso se forma un precipitado marrón oscuro que corresponde a la fracción de las PACs.
- Recoger el precipitado resultante y secar por flujo de aire caliente hasta un contenido de disolvente inferior a 10% en peso.
- Someter el producto obtenido en la etapa anterior a 120-180ºC durante al menos 3 minutos y posteriormente dejarlo enfriar.
- Obtener un producto final con aspecto vítreo, de color marrón y que se rompe fácilmente. Una vez frío, se muele en molino de martillo con una malla inferior a 0,5mm hasta obtener un tamaño de partícula inferior a 180µm. Finalmente se recupera el producto de color marrón, amorfo, apenas soluble en agua y con un elevado contenido en fibra insoluble y PACs de elevado peso molecular.

3.2. Técnicas *in vitro*

Determinación de la cinética de la enzima α-glucosidasa actividad maltasa.

Este ensayo se basa en propiedad que presentan las glucosidasas para hidrolizar enlaces glucosídicos y originar hidratos de carbonos más simples. Con dicha técnica estudia exclusivamente la actividad maltasa de la enzima α-glucosidasa al utilizar como sustrato maltosa, pudiéndose cuantificar la producción de glucosa a partir de dicho disacárido.

Se evaluó la actividad de la α-glucosidasa (maltasa) según el método descrito por Garcimartín y cols. (2015a). Para ello se preparó una solución de enzima a partir de 1g de polvo intestinal de rata (Sigma-Aldrich, Madrid, España) en tampón maleato (pH 6,0). La maltosa se utilizó como sustrato de la reacción enzimática, seleccionándose 40 y 80 mg/mL como concentraciones de estudio. Se prepararon diferentes concentraciones de CFE (0,5, 1, 2 y 5mg/mL) en tampón maleato. Se adicionaron a la mezcla de reacción 75µL de extracto enzimático, 75µL de CFE y 150µL de maltosa. Las muestras se mantuvieron en agitación constante a 37ºC durante 90 min en un baño termostatizado (Unitronic 320 OR, Selecta, Barcelona, España), recogiéndose alícuotas cada 15 min para determinar la cantidad de glucosa producida mediante un kit colorimétrico Glucosa-TR (Spinreact, Barcelona, España). Se utilizó tampón maleato como control negativo y acarbosa (Sigma-Aldrich) 1 mg/L como control

positivo. Las determinaciones se realizaron por triplicado y se expresaron en mmol/L de glucosa producida por minuto.

Se estudió el mecanismo de inhibición del CFE sobre la enzima α-glucosidasa actividad maltasa de acuerdo al protocolo descrito previamente. Para ello se emplearon concentraciones crecientes de maltosa (10, 20, 40 y 80mg/mL). Dado que todas las reacciones mostraron una cinética Michaeliana, se calculó la actividad para cada concentración de sustrato y de CFE. Posteriormente se realizó un ajuste por Lineweaver–Burk (1/v vs. 1/[S]), donde v hace referencia a la velocidad y [S] es la concentración de sustrato de cada uno de los compuestos de estudio, obteniéndose la constante de afinidad (Km) y la velocidad máxima (Vmax) (Lineweaver & Burk, 1934). Este método se empleó y citó en el **artículo 1**.

Difusión de glucosa

Se midió de acuerdo al protocolo descrito por Schultz-Moreira y cols. (2014a) con pequeñas modificaciones. Dicha técnica se fundamenta en la difusión de glucosa a través de una membrana de diálisis. Se introdujo en una membrana de diálisis de 7 cm × 10 mm con un tamaño de poro de 25Å (14.000 Da) (Sigma-Aldrich, Madrid, España) una solución de glucosa (40mg/mL) (Sigma-Aldrich), a la cual se adicionó diferentes concentraciones (0, 0,5, 1, 2 y 5mg/mL) de CFE o PBS como control negativo. Los extremos de las membranas se cerraron cuidadosamente y se colocaron en recipientes con 100 mL de solución salina al 0,9% peso/volumen. Los recipientes se mantuvieron a 37ºC y fuerte agitación durante 4h, obteniéndose alícuotas de la solución de NaCl cada media hora. La cantidad de glucosa difundida se cuantificó utilizando el kit colorimétrico Glucosa-TR (Spinreact). Los resultados se expresaron en mmol/L de glucosa respecto al tiempo y también se calculó el área bajo la curva (AUC) después de 4h, considerando los valores de glucosa difundida cada media hora para cada tiempo. Todos los ensayos se realizaron por cuadruplicado. El método descrito se empleó y citó en el **artículo 1**.

Determinación de la actividad lipasa pancreática

La propiedad que presenta la lipasa pancreática para descomponer triglicéridos en lípidos más sencillos (diglicéridos, monoglicéridos y AGL), permite evaluar su actividad enzimática utilizando un método colorimétrico. De acuerdo al método descrito por Winker et al., se preparó una solución enzimática de lipasa pancreática porcina (10mg/mL) (Sigma-Aldrich), la cual se mezcló con diferentes concentraciones de CFE (1, 2,5, 5 y 10mg/mL) en PBS durante 10 min a 37ºC. Pasado este tiempo se adicionó

p-nitrofenilpalmitato como sustrato, en presencia de desoxicolato de sodio y goma arábiga, y se mantuvieron en agitación durante 20min a 37ºC. La actividad lipasa se midió espectrofotométricamente a 410nm (SPECTROstar Nano, BMG LABTECH, Offenburg, Alemania). Los experimentos se realizaron por triplicado y los datos se expresaron como porcentaje de actividad con respecto al control. El método descrito se puede localizar en el **artículo 2**.

Caracterización del grado de lipolisis de las digestiones in *vitro*

Para la extracción de la materia grasa de las digestiones *in vitro* se empleó el método de Bligh & Dyer (1959). El remanente de la digestión enzimática se mezcló dos veces con una mezcla cloroformo/metanol (1:1, v/v) (Sigma-Aldrich). Posteriormente se recogió la fase orgánica y se purificó usando una mezcla de solución de cloroformo/metanol/NaCl al 0,9% (vol:vol, 3/48/47). El resultante se deshidrató filtrándolo a través de sulfato de sodio anhidro (Merck, Madrid, España), se evaporó en rotavapor a 40ºC y se sometió a corriente de nitrógeno durante 3min hasta obtener la fase lipídica aislada.

Con la finalidad de conocer la composición lipídica con mayor precisión, se empleó la técnica desarrollada por Dobarganes y cols. (2000) y aceptadas como oficiales por la Unión Internacional de Química Pura y Aplicada (IUPAC, de sus siglas en inglés *International Union of Pure and Applied Chemistry*). Para ello, se realizó una separación mediante cromatografía líquida de alta eficacia por exclusión por tamaño molecular (HPSEC, por sus siglas en inglés *High Performance Size Exclusion Chromatography*). Se aplicaron 15mg de grasa extraída diluida en 1mL de tetrahidrofurano en HPSEC (serie Agilent 1100, Madrid, España) utilizando 20 µl como volumen de inyección. Un detector de índice de refracción (Agilent Technologies 1260 infinity, Madrid, España) y dos columnas de poliestireno-divinilbenzeno (PLgel) (Agilent, Bellefonte, PA, EE.UU.) de 300 mm x 7,5 mm (5 µm de tamaño de partícula), con 0,01 y 0,05 µm de tamaño de poro, conectadas en serie, termostatizadas a 40ºC. Como fase móvil se utilizó tetrahidrofurano en régimen isocrático a un flujo de 1mL/min. Los triglicéridos (TG), diglicéridos (DG), monoglicéridos (MG) y ácidos grasos libres (AGL) se cuantificaron como g/100g de muestra. Todos los análisis se realizaron por duplicado. El método descrito se utilizó e incluyó en el **artículo 3**.

3.3. Técnicas *in vivo*

3.3.1. Diseño experimental del estudio *in vivo* a corto plazo

Veinticuatro ratas Wistar macho (Harlan S.L., Barcelona, España) de aproximadamente 2 meses de edad y 200g de peso, se distribuyeron en 4 grupos de acuerdo a las dosis de CFE a estudiar: a) control, b) CFE 25mg/kg p.c.; c) CFE 50mg/kg p.c y d) CFE 150mg/kg p.c. Este estudio se subdividió en dos experimentos de digestibilidad, uno agudo (administración de una dosis única de CFE y agua en el grupo control) y otro subcrónico (administración de CFE y agua en el grupo control durante una semana). Para el estudio postprandial se administró por canulación 1mL de aceite de oliva y 1mL de una solución de glucosa 0,5g/mL (Sigma-Aldrich) a todos los animales. Los grupos con CFE recibieron este compuesto incorporado en la mezcla de aceite y glucosa. El esquema del diseño experimental se representa en la figura 12 (**Artículos 1 y 2**).

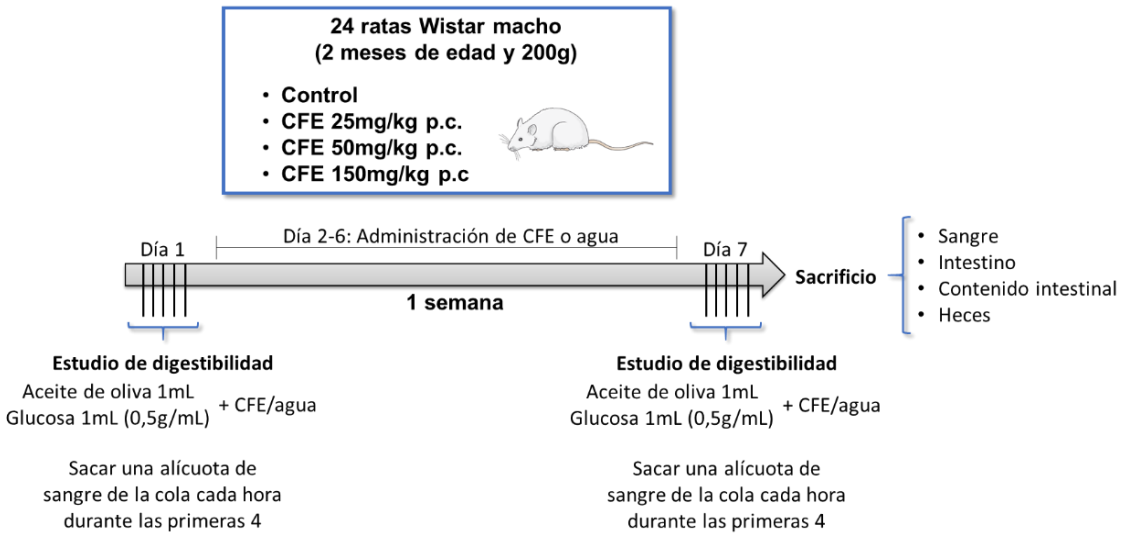

Figura 12. Diseño experimental del estudio *in vivo* a corto plazo.

3.3.2. Formulación de los reestructurados cárnicos

La preparación de los reestructurados cárnicos (RC) se realizó según el protocolo descrito por Schultz-Moreira y cols. (2010b). Como ingrediente principal se utilizó carne picada magra mixta (50% cerdo: 50% vacuno), la cual se mezcló de forma homogénea con manteca de cerdo durante 1 minuto en una picadora-homogenizadora conectada a un baño de refrigeración (2°C) (Stephan Universal Machine UM5, Stephan u. Söhne GmbH and Co., Hameln, Alemania). Para el cárnico enriquecido con CFE (RC-CFE) se adicionaron 4g/kg de dicho extracto, mientras que en el cárnico

control (RC-C) esta cantidad fue sustituida por celulosa, con la finalidad de mantener el mismo aporte de fibra dietética. Una vez elaborados los RC, se liofilizaron en un liofilizador LyoAlfa 10 (Telstar, Terrassa, España) durante 48h. Los productos liofilizados se molieron en una picadora refrigerada (Stephan Universal Machine UM5) durante 2 minutos hasta conseguir un polvo fino homogéneo (Tabla 6).

Tabla 6. Composición de los reestructurados cárnicos

Componentes del RC	**RC-C**	**RC-CFE**
Proteínas, %	13,1	13,1
Grasas, %	38,3	38,3
Agua, %	46,9	46,9
Colesterol, g/kg	0,74	0,74
AGS:AGM:AGP ratio	41,2/43,5/8,5	41,2/43,5/8,5
Ingredientes, g/kg		
Carne picada mixta	663,1	663,1
Manteca de cerdo	331,1	331,1
Sodio	0,5	0,5
Tripolifosfato de sodio	0,1	0,1
Nitrito sódico	1,2	1,2
Celulosa	4	0
CFE	0	4

AGM, ácidos grasos monoinsaturados; AGP, ácidos grasos poliinsaturados; AGS, ácidos grasos saturados; CFE, extracto de algarroba; RC, reestructurado cárnico; RC-C, reestructurado cárnico control; RC-CFE, reestructurado cárnico enriquecido con CFE.

3.3.3. Dietas empleadas en el estudio *in vivo* crónico

La inclusión de los RC en las dietas se realizó según lo descrito en la Tabla 7, elaborando una mezcla homogénea con un 70% de dieta semisintética (Panlab S.L., Barcelona, España, referencia U8959 versión 180) y un 30% de RC liofilizado. El porcentaje de cárnico fue seleccionado de acuerdo a otros estudios del grupo AFUSAN, con la intención de imitar el elevado consumo de carne en los países occidentales (González-Torres y cols., 2016; Santos-López y cols., 2017). Para una mejor comprensión se describe la composición de las dietas utilizadas en el desarrollo de ambos modelos de DMT2.

- Modelo murino de estadio inicial de DMT2: en este estudio se formularon una dieta control (C) alta en grasas saturadas y con un 30% de RC-C liofilizado; y una dieta de tratamiento (CE), alta en grasas saturadas y con un 30% de RC-CFE. Ambas dietas presentan el mismo perfil calórico, con aproximadamente un 50% de las kcal totales procedentes de las grasas

(del total de las cuales un 39% eran grasas saturadas), un 35% de los hidratos de carbono y un 15% de las proteínas (Tabla 7).

- Modelo murino de estadio tardío de DMT2: para este modelo experimental se elaboraron dos dietas: a) dieta Chol, alta en grasas saturadas y colesterol (1,2% de colesterol + 0,2% de ácido cólico) a la que se incorporó un 30% RC-C; b) dieta Chol-CFE, formulada a partir de dieta con alto contenido en grasas saturadas y colesterol (1,2% de colesterol + 0,2% de ácido cólico) y un 30% de RC-CFE. Un 50% de las kcal totales de la dieta provenían de las grasas (del total de las cuales un 39% eran grasas saturadas), un 35% de los hidratos de carbono y un 15% de las proteínas (Tabla 7).

Tabla 7. Composición de las dietas experimentales para el experimento crónico

	Dieta			
	Modelo de DMT2 inicial		**Modelo de DMT2 tardío**	
Componentes de la dieta	**C**	**CE**	**Chol**	**Chol-CFE**
Proteínas, % energía	14,15	14,15	14.0	14.0
Grasas, % energía	49,42	49,42	49.0	49.0
AGS:AGM:AGP ratio	2,76/2,99/1	2,76/2,99/1	2.10/2.32/1	2.10/2.32/1
Contenido energético*, MJ/kg	27,52	27,52	20.36	20.36
Ingredientes, g/kg				
Sacarosa	68,25	68,25	68,25	68,25
Almidón de maíz	286,13	286,13	275,73	275,73
Caseína	94,25	94,25	94,25	94,25
L-Cisteína	2,02	2,02	2,02	2,02
Maltodextrina	94,25	94,25	94,25	94,25
Celulosa	48,86	48,86	48,86	48,86
PM 205B SAFE	50,05	50,05	50,05	50,05
PV 200 SAFE	7,15	7,15	7,15	7,15
Aceite de soja	47,91	47,91	47,91	47,91
Colesterol	0	0	9,1	9,1
Ácido cólico	0	0	1,3	1,3
Cárnico enriquecido	301,14	301,14	301,14	301,14

C, dieta con cárnico control; CE, dieta con cárnico enriquecido en CFE; AGM, ácidos grasos monoinsaturados; AGP, ácidos grasos poliinsaturados; AGS, ácidos grasos saturados; PM 205B SAFE, mezcla de minerales; PV 200 SAFE, mezcla de vitaminas. Chol, dieta hipercolesterolémica con cárnico control + 1.4% de colesterol y 0.2% de ácido cólico; Chol-CFE, dieta hipercolesterolémica con cárnico enriquecido con CFE + 1.4% de colesterol y 0.2% de ácido cólico. El cárnico control se formuló con celulosa (4g/kg) y el cárnico enriquecido en CFE se elaboró con extracto de algarrobo (4g/kg), con el fin de mantener en las dietas el mismo aporte de fibra dietética.
*Datos calculados considerando como equivalente de energía para hidratos de carbono 16.73 kJ/g (4 kcal/g); grasa, 37.65 kJ/g (9 kcal/g); proteína 16.73 kJ/g (4 kcal/g).

3.3.4. Diseño experimental del estudio *in vivo* crónico

3.3.4.1. Modelo murino de estadio inicial de DMT2

Se emplearon 2 lotes de ratas Wistar macho (Harlan S.L) de dos meses de edad y aproximadamente 200g de peso (n=8 cada uno de ellos) en las que se indujo DMT2 con dieta alta en grasas saturadas. El primer grupo, designado como C, recibió dieta alta en grasas saturadas + cárnico control; mientras que el segundo grupo, denominado CE, fue alimentado con dieta alta en grasas saturadas + cárnico enriquecido en CFE. Recibieron esta alimentación durante 8 semanas de estudio, con la finalidad de que presentaran una fisiopatología similar a una etapa inicial de DMT2, con hiperglucemia e hiperinsulinemia. Este modelo animal está descrito en el **artículo 7**. La figura 13 muestra el diseño experimental del modelo de estado inicial de DMT2

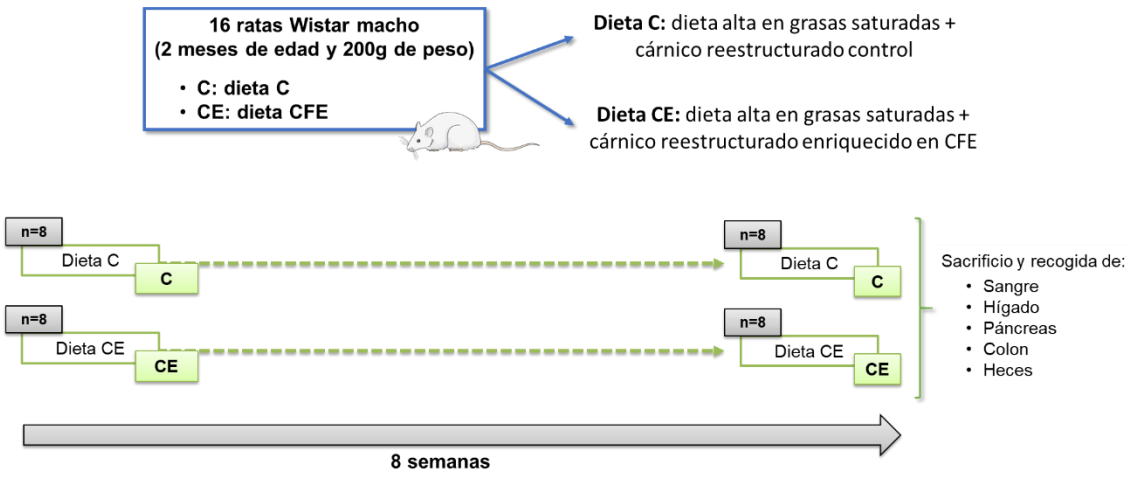

Figura 13. Diseño experimental del estudio *in vivo* en un modelo murino de estado inicial de DMT2

3.3.4.2. Modelo murino de estadio tardío de DMT2

24 ratas Wistar macho (Harlan S.L) de aproximadamente 2 meses de edad y 200g de peso, fueron divididas en 3 grupos de acuerdo la dieta a recibir: a) DMT2 control (D), b) grupo que consumió CFE como tratamiento preventivo CFE (ED) y c) grupo alimentado con CFE como tratamiento terapéutico (DE). Todos los grupos recibieron dieta con alto contenido en grasas saturadas/alta en colesterol (1,2% de colesterol + 0,2% de ácido cólico), a la que se incorporó el reestructurado cárnico control (Dieta Chol) o enriquecido en CFE (Dieta CFE). El grupo ED fue alimentado con dieta CFE durante todo el experimento, mientras que los grupos D y DE comenzaron con la dieta Chol y el cárnico control o enriquecido en CFE, respectivamente, durante 3 semanas.

En esta tercera semana se indujo diabetes mediante inyección intraperitoneal de estreptozotocina (STZ, 65mg/kg p.c.) y nicotinamida (NAD, 225mg/kg p.c.) (Sigma-Aldrich) en todos los grupos. El estado diabético se confirmó mediante la medición de glucemia plasmática a las 72h. Una vez comprobada la hiperglucemia, el grupo DE recibió dieta CFE durante 5 semanas (tratamiento terapéutico), mientras que los grupos D y ED mantuvieron la misma alimentación durante todo el experimento. Tras 8 semanas, los animales fueron sacrificados, recogiéndose los diferentes órganos y la sangre para su estudio. Con este diseño se obtuvo un modelo de DMT2 similar a un estado avanzado de la patología, con hiperglucemia e hipoinsulinemia. Este modelo animal se ha empleado en los **artículos 8, 9, 10 y 11**. Dada la complejidad del diseño experimental, se ha incluido un resumen en la figura 14.

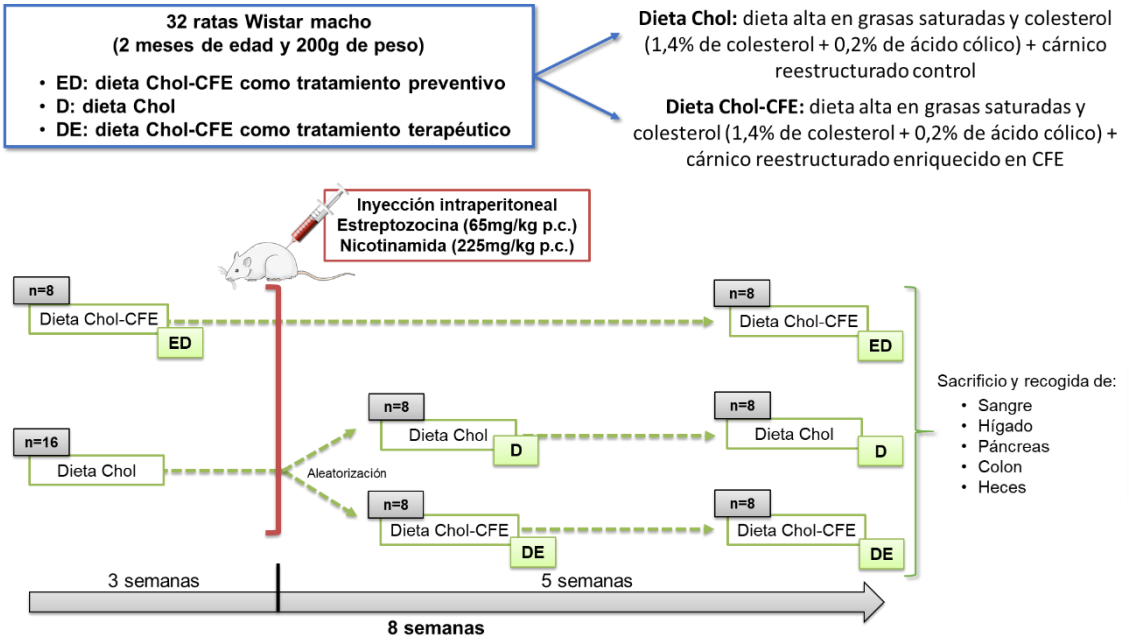

Figura 14. Diseño experimental del estudio *in vivo* en un modelo murino de estadio tardío de DMT2

Todos los experimentos se realizaron conforme a la Directiva 86/609/CEE, del 24 de noviembre de 1986 (modificada por la Directiva 2003/65/CEE, del 22 de julio de 2003), relativa a la protección de los animales en investigación científica. Este estudio fue aprobado por el Comité Asesor de Ciencia y Tecnología (AGL2014-53207-C2-2-R), y por el Comité de Ética de la Universidad Complutense de Madrid. En ambos modelos animales los animales tuvieron acceso a comida y agua *ad libitum* y se mantuvieron bajo condiciones controladas (22,3 ± 1,8°C y ciclo de luz-oscuridad de 12h) en el Centro de Experimentación Animal de la Universidad de Alcalá (nº registro ES280050001165).

3.3.4.3. Determinaciones plasmáticas

Las determinaciones bioquímicas se realizaron en el plasma de los animales de estudio, el cual se obtuvo centrifugando la sangre extraída a 986 *g* durante 10 min.

Glucosa. Las concentraciones de glucosa plasmática se determinaron mediante el kit Glucosa-TR (Spinreact). El método consiste en la reacción de la glucosa oxidasa que cataliza la oxidación de glucosa a ácido glucónico. El peróxido de hidrógeno resultante se detecta mediante un aceptor cromogénico de oxígeno (fenol-ampirona) en presencia de peroxidasa, que origina un color rosa y es medido espectrofotométricamente a 505nm (SPECTROstar Nano). La intensidad del color formado es proporcional a la concentración de glucosa presente en la muestra ensayada (**Artículos 1, 7, 8 y 9**).

Triglicéridos. Se cuantificó la trigliceridemia mediante el kit colorimétrico Triglicéridos-LQ (Spinreact). Los triglicéridos incubados con lipoprotein-lipasa (LPL) liberan glicerol y ácidos grasos libres. El glicerol es fosforilado por la enzima glicerolfosfato deshidrogenasa y ATP en presencia de glicerol quinasa originando glicerol-3-fosfato y adenosina-5-difosfato. El glicerol-3-fosfato es posteriormente convertido a dihidroxiacetona fosfato y peróxido de hidrogeno por la enzima GPO. Finalmente, el peróxido de hidrogeno reacciona con 4-aminofenazona y p-clorofenol, reacción catalizada por la peroxidasa dando una coloración roja medible espectrofotométricamente a 505nm (SPECTROstar Nano) (**Artículos 2, 7 y 8**).

Colesterol. La cuantificación de los niveles de colesterol se realizó mediante el kit Colesterol-LQ (Spinreact). El colesterol (esterificado + libre) origina, tras una serie de reacciones acopladas, quinonimina, un compuesto coloreado directamente proporcional a la cantidad de colesterol de la muestra. La intensidad del color se cuantificó espectrofotométricamente a 505 nm en un lector placas (SPECTROstar Nano) (**Artículos 2, 7 y 8**).

Fosfolípidos. La determinación de la fosfolipemia se realizó mediante el kit colorimétrico Fosfolípidos CHO-POD (Spinreact). Los fosfolípidos son hidrolizados por la enzima fosfolipasa D, y la colina liberada es secuencialmente oxidada por la colina oxidasa a betaína, con la simultánea producción de peróxido de hidrógeno. En presencia de peroxidasa, el peróxido de hidrógeno acopla oxidativamente a la 4-aminofenazona (4-AF) y al diclorofenol formando una quinonamina coloreada cuantificable a 505 nm (SPECTROstar Nano) (**Artículos 7 y 8**).

Insulina. Se utilizó un kit comercial tipo ELISA para cuantificar los niveles plasmáticos de insulina (Rat insulin ELISA Kit, ELR-Insulin, RayBiotech, Inc., EE.UU.), siguiendo las instrucciones del fabricante (**Artículos 7 y 9**).

Índices de resistencia a la insulina (HOMA-IR) y actividad pancreática (HOMA-β). El cálculo del HOMA-IR y HOMA-β se realizó de acuerdo a lo descrito por Gesteiro y cols. (2012) y Song y cols. (2007), respectivamente. Estos índices definen la relación entre la glucemia basal y los niveles de insulina, permitiendo discernir entre la RI o la funcionalidad pancreática en función de la ecuación aplicada. Los cálculos se obtienen con las siguientes fórmulas: HOMA-IR = insulina en ayunas (μUI/mL) × glucosa en ayunas (mmol/L)/22,5; HOMA-β = 20 × insulina en ayunas (μIU/mL)/glucosa en ayunas (mmol/L)–3,5 (**Artículos 7 y 9**).

Aislamiento y caracterización de lipoproteínas: Las diferentes fracciones de lipoproteínas se obtuvieron a partir de 2mL de plasma mediante ultracentrifugación en gradiente salino según el método de Terpstra y cols. (1981), aplicando las modificaciones descritas por Olivero-David y cols. (2011). Para la separación de las fracciones lipoproteicas utilizaron tubos Ultra-clear (Beckman) los cuales se rotularon con marcas de volumen de 1mL y un rotor SW-40.1. (Beckman L8-70M, Palo Alto, California). Los tubos se centrifugaron durante 21h 40 min a 272.000 *g* (40.000 rpm) a 4ºC. El aislamiento de las fracciones de lipoproteínas se realizó usando un cortatubos (Beckman) teniendo en cuenta el intervalo de densidad convencional para ratas de las diferentes clases de lipoproteínas [VLDL ($\rho 20<1{,}0063$ g/mL), IDL ($1{,}0063<\rho 20<1{,}019$), LDL ($1{,}019<\rho 20<1{,}057$) y HDL ($1{,}057<\rho 20<1{,}21$ g/mL)].

Se cuantificaron los triglicéridos, colesterol total y fosfolípidos de las diferentes fracciones de lipoproteínas (VLDL, IDL, LDL y HDL) utilizando los kits colorimétricos Triglicéridos-LQ, Colesterol-LQ y Fosfolípidos CHO-POD (Spinreact), descritos previamente. El contenido de proteínas de las lipoproteínas aisladas se determinó mediante el método de Bradford (1976) (**Artículos 7 y 8**).

Arilesterasa: La actividad AE se determinó en plasma y en extractos hepáticos de los animales de estudio de acuerdo al método de Nus y cols. (2008). Para su determinación se utilizó SBF (*simulated body fluid*) como buffer y fenilacetato como sustrato. Se evaluó la cinética de la reacción de AE a 270nm en cubetas de cuarzo Lightpath de 10 mm controladas termostáticamente a 37ºC en un espectrofotómetro (SPECTROstar Nano). Se utilizaron blancos sin plasma para corregir la hidrólisis espontánea de fenilacetato que tiene lugar en el tampón. Los

resultados se expresaron como mmol de fenol formado a partir de fenilacetato por minuto, los cuales fueron normalizados por litro de plasma o por mg de proteína en el caso de los extractos hepáticos (**Artículos 7 y 8**)

Superóxido dismutasa (SOD): La actividad de la SOD se determinó según el método del azul de nitro-tetrazolio (NBT por sus siglas en inglés, Nitroblue Tetrazolium) con ligeras modificaciones. Esta técnica se basa en la generación de radical superóxido ($O2^-$) a partir de la auto-oxidación del clorhidrato de hidroxilamina. En el medio de reacción, el NBT es reducido a nitrito y éste reacciona con EDTA (sal disódica dihidratada del ácido etilendiaminotetraacético) formando un complejo coloreado que se cuantifica midiendo la absorbancia a 560 nm cada minuto durante 10min a 37°C (SPECTROstar Nano). Los resultados se expresaron en UI/mg de proteína (**Artículo 11**).

Catalasa (CAT): La actividad de la CAT fue determinada en plasma y extractos hepáticos según el método descrito por Aebi (1984), el cual se basa en la reacción de la descomposición del peróxido de hidrogeno por acción enzimática. La técnica consiste en monitorizar la absorbancia a 260nm (SPECTROstar Nano) a medida que se descompone el sustrato (peróxido de hidrógeno) por la CAT presente en la muestra. Las lecturas se realizaron cada 30s durante 10min a 37°C. Los resultados se expresan en UI/mg de proteína (**Artículo 11**).

Glutation reductasa (GR). Se evaluó la actividad de la GR de acuerdo con el método de Carlberg & Mannervik (1985). El fundamente de esta técnica se basa en determinar la oxidación de NADPH a $NADP^+$ durante la reducción del glutation oxidado (GSSG) a glutation reducido (GSH) (ambos de Sigma-Aldrich) mediante la disminución de la absorbancia a 340nm y 37°C. Los resultados se expresaron como nmol de NADPH oxidado a $NADP^+$ por minuto/mg de proteína (**Artículo 11**).

Glutation peroxidasa (GPx). La actividad de la GPx se analizó según el protocolo descrito por Flohé et al con algunas modificaciones (Flohé & Günzler, 1984). Dicha técnica se basa en cuantificar la oxidación de NADPH cuando el GSSG se reduce mediante GR en presencia de hidroperóxido de cumeno como sustrato (Sigma-Aldrich) (26). La disminución de la densidad óptica debido a la oxidación de NADPH a 340nm se leyó en un lector de placas de fluorescencia (FLUOstar Omega, BMG LABTECH, Offenburg, Alemania) a intervalos de 1min durante 10min. Los resultados se expresaron como nmol de NADPH oxidado a $NADP^+$ por minuto/mg de proteína (**Artículo 11**).

Glutation reducido (GSH) y oxidado (GSSG): Se cuantificaron los niveles de GSH y GSSG siguiendo el método de Senft y cols. (2000), utilizando como sonda fluorescente el orto-ftalaldehído (OPA). El plasma y los extractos hepáticos y colónicos se desproteinizaron en tampón RQB (Redox Quenching Buffer) con un 5% de ácido tricloroacético (TCA-RQB), se homogeneizaron mediante sonicación a 4ºC y se centrifugaron durante 10min a 14.000 *g*. Se recogieron los sobrenadantes y la reacción se llevó a cabo en una placa de 96 pocillos en la que se añadió la muestra, RQB, N-etilmaleimida 3,15mM, tampón fosfato 0,1M y OPA (5 mg/mL en metanol) (todos de Sigma-Aldrich) para la determinación de GSH. Los niveles de GSSG se cuantificaron después de añadir ditionito 95,62mM para reducir todo el GSSG presente en la muestra a GSH. La fluorescencia se midió a λexc = 360nm y λem = 460nm (FLUOstar Omega). Las lecturas fluorométicas se extrapolaron a una curva estándar de GSH y los resultados se expresaron como nmol/mg de proteína. Además, se calculó el índice redox, parámetro indicativo del estado de oxidación, según la siguiente ecuación: IR=GSSG/(GSH+GSSG) (**Artículo 11**).

3.3.4.4. Western Blot

Se obtuvieron extractos proteicos totales y de fracciones subcelulares (citosólicas, mitocondriales y nucleares) a partir del tejido hepático y colónico (mucosa proximal y distal). Los tejidos se homogeneizaron con tampón de lisis (en función del proceso de extracción a seguir) y cóctel inhibidor de proteasas al 0,01% (Sigma-Aldrich). Posteriormente, las muestras se separaron mediante electroforesis en gel de poliacrilamida/dodecil sulfato de sodio (SDS-PAGE) del 8-15% a 150V. Una vez acabado este proceso, se realizó la transferencia húmeda (Mini Trans-Blot Cell, Bio-Rad, Madrid, España) o seca (Trans-Blot Turbo, Bio-Rad,) a una membrana de fluoruro de polivinilideno (PVDF) (GE Healthcare, Madrid, España). Las membranas se bloquearon con albúmina de suero bovino al 5% durante 1h, y se incubaron durante la noche a 4ºC con los anticuerpos primarios correspondientes (Tabla 8). La hibridación de anticuerpos se reveló incubando las membranas con los anticuerpos secundarios apropiados conjugados con peroxidasa durante 1h a temperatura ambiente. La señal de quimioluminiscencia se detectó utilizando el kit ECL Select-kit (GE Healthcare) de acuerdo a las instrucciones del fabricante y se detectó en ImageQuant LAS 500 (GE Healthcare). La densidad de banda se cuantificó utilizando el programa Fiji ImageJ v1.52j (National Institute of Health, EE.UU.) (**Artículos 1, 7, 8, 9 y 11**).

3.3.4.5. Inmunohistoquímica

Los cortes de hígado, páncreas y colon (proximal y distal) se fijaron en paraformaldehído al 4% en tampón fosfato 0,1M, pH 7,4, se deshidrataron y se embebieron en parafina. Posteriormente, las secciones de tejido se desparafinaron y se inactivó la peroxidasa endógena con peróxido de hidrógeno al 3%. Las secciones se incubaron durante la noche a 4°C con los anticuerpos primarios correspondientes en cada caso y que se detallan en la Tabla 8. Pasado este tiempo, se hicieron varios lavados y las secciones se cubrieron con el anticuerpo secundario biotinilado apropiado. La tinción inmunohistoquímica se realizó utilizando peroxidasa de rábano conjugada con estreptavidina-biotina (Sigma-Aldrich) y se visualizó mediante incubación con 3, 3'-diaminobencidina (DAB) (Sigma-Aldrich). Las secciones fueron teñidas con hematoxilina de Harris, deshidratadas y montadas sobre portas. Para su análisis se utilizaron scores o se cuantificó el porcentaje de área DAB positiva mediante el software Fiji ImageJ v1.52j (National Institute of Health) (**artículos 7, 8, 9, 10 y 11**)

Tabla 8. Anticuerpos primarios empleados en Western Blot e inmunohistoquímica

Proteína		**Referencia**
AKT2	AKT isoforma 2	sc-81148
pAKT2^{ser473}	Fosfo-AKT isoforma 2 (serina 473)	sc-514032
CAT	Catalasa	sc-34282
ChREBP	Proteína de unión al elemento de respuesta a carbohidratos	sc-33764
GLUT-2	Transportador de glucosa tipo 2	sc-518022
GPx	Glutation peroxidasa	sc-22146
GR	Glutation reductasa	sc-32886
GSK3β	Glucógeno sintasa quinasa 3β	sc-377213
pGSK3β^{ser9}	Fosfo-Glucógeno sintasa quinasa 3β (serina 9)	sc-373800
Insulina	Insulina	sc-8033
InsRβ	Receptor de insulina subunidad β	sc-57342
LDLr	Receptor para lipoproteínas de baja densidad	sc-18823
LXR	Receptor X hepático	sc-377260
pNrf2^{ser40}	Fosfo-factor nuclear eritroide 2 (serina 40)	SAB-12811
Occludina	Occludina	sc-133256
PCNA	Antígeno nuclear de células en proliferación	sc-7907
PI3K-C2α	Fosfatidilinositol 3-quinasa	sc-365290
SGLT1	Cotransportador de sodio-glucosa tipo 1	sc-98974
SOD1	Superóxido dismutasa 1	sc-11407
SOD2	Superóxido dismutasa 2	sc-30080
SREBP-1c	Proteína de unión al elemento regulador del esterol 1c	sc-8984
Zonulina-1	Zonulina-1	sc-33725
β-Actina	β Actina	sc-47778

SAB, Signalway Antibody (Baltimore, Maryland, EE.UU.); sc; Santa Cruz Biotechnology (Quimigen, Madrid, España)

3.3.4.6. Estudio histológico

Tal y como se ha comentado previamente, los cortes histológicos se fijaron en paraformaldehído al 4% en tampón fosfato 0,1M, pH 7,4, se deshidrataron y se embebieron en parafina.

Hematoxilina y eosina (H&E): es una técnica histológica clásica basada en dos reacciones, una tinción del núcleo por un colorante básico (hematoxilina), y una tinción del citroplasma por un colorante ácido (eosina). Permite un buen contraste de las preparaciones microscópicas facilitando su observación. Esta tinción se utilizó para el estudio del páncreas, hígado y colon.

Tinción de PAS (Periodic Acid-Schiff): esta técnica permite detectar hidratos de carbono en los tejidos, tanto glucógeno como mucopolisacáridos. Se basa en la oxidación de las uniones carbono-carbono de los azúcares para formar grupos aldehídos el ácido peryódico. A continuación se trata con el reactivo de Schiff que reacciona cuando existen dos grupos aldehídos contiguos, dando lugar a una coloración rojo-púrpura. Esta tinción se empleó en la determinación del glucógeno hepático y las células caliciformes colónicas.

Las secciones hepáticas, pancreáticas y colónicas se tiñeron con H&E y/o PAS de acuerdo con los métodos de rutina. Las imágenes de las secciones se estudiaron y cuantificaron con un aumento de ×200 o ×400 utilizando un microscopio Leica DM LB2 equipado con una cámara digital Leica DFC 320 (Leica Microsystems, L'Hospitalet del Llobregat, España) y se realizó un análisis morfométrico con el software Fiji ImageJ v1.52j (Instituto Nacional de Salud) (**artículos 9, 10 y 11**).

3.3.4.7. Peroxidación lipídica

Para determinar la oxidación hepática se utilizó la técnica de TBARS (de sus siglas en inglés, *thiobarbituric acid reactive substances*) según lo descrito por Uchiyama & Mihara (1978). Esta técnica se basa en la reacción del malondialdehído (MDA) y el ácido tiobarbitúrico a temperatura elevada, originando un complejo coloreado directamente proporcional a la peroxidación lipídica. Para ello, se homogenizaron 50mg de hígado en tampón fosfato 50mM y se centrifugaron a 4000 *g* durante 20 min a 4ºC. El sobrenadante resultante se recogió y se mezcló con una solución de ácido tiobarbitúrico, la cual se mantuvo a 100ºC durante 45min. El contenido de MDA se cuantificó en un lector de placas de fluorescencia (FLUOstar Omega) a λexc = 485nm y λem = 520nm, extrapolando las lecturas a la curva estándar

de MDA generada por hidrólisis catalizada por ácido de 1,1,3,3-tetrametoxipropano (Sigma-Aldrich) (**artículos 7 y 8**).

3.3.4.8. Determinación del glucógeno hepático

El glucógeno hepático se determinó mediante una ligera modificación del método de Morales y cols. (1973). 500mg de hígado se digirieron hirviéndolas en KOH al 30% durante 20 min. Posteriormente se enfriaron y se añadieron 3mL de etanol al 95%, una gota de acetato de amonio 1M y se llevaron a ebullición. Pasados los 20min, los tubos se enfriaron y centrifugaron a 3000 *g* durante 10min, descartándose el sobrenadante. A continuación, los residuos se lavaron tres veces, se añadieron 4mL de reactivo de antrona (Sigma-Aldrich) y se mantuvieron en hielo. Una vez fríos, los tubos se incubaron durante 20min a 100°C para el desarrollo del color y se midió la absorbancia a 640 nm en un lector de microplacas (SPECTROstar Nano). Se utilizaron soluciones de glucosa y agua destilada como blanco y estándar, respectivamente. Los resultados se expresaron como mg de glucógeno/g de tejido (**artículo 9**).

3.3.4.9. Valoración de la apoptosis

Se realizó un ensayo de fragmentación del ADN (TUNEL, de sus siglas en inglés *terminal deoxynucleotidyl transferase dUTP nick end labelling*) para determinar los colonocitos apoptóticos de acuerdo a López-Oliva y cols. (2013). El método se basa en la capacidad de la desoxinucleotidil transferasa para marcar los extremos romos de las roturas de ADN de doble hebra mediante la adición de desoxinucleótidos marcados. El índice TUNEL (%) se calculó como el número de células apoptóticas x 100/número total de células/altura de la cripta. Para la cuantificación del índice TUNEL, se examinaron y contaron al menos 50 criptas perpendiculares bien orientadas para cada animal con un aumento de ×400 utilizando un microscopio Leica DM LB2 equipado con una cámara digital Leica DFC 320 (Leica Microsystems) (**artículo 10**).

3.3.4.10. Extracción de ADN fecal. Análisis de la microbiota (qPCR).

Las heces extraídas directamente del colon distal se recolectaron en tubos estériles y se congelaron inmediatamente a -80°C hasta el momento del análisis. Se extrajo ADN bacteriano de 180-220 mg de cada muestra fecal utilizando un protocolo optimizado que incluía la homogeneización con perlas de vidrio en el equipo FastPrep (MP Biomedicals, California, EE.UU.) y la extracción de ADN con columnas comerciales QIAamp DNA Stool Mini Kit (Qiagen NV, Venlo, Holanda) siguiendo las instrucciones del fabricante. El ADN extraído se mantuvo congelado a -80ºC hasta su

uso posterior. La concentración de ADN se midió en un espectrofotómetro Nanodrop ND-1000 (Thermo Fisher Scientific, Wilmington, EE.UU.).

Para el análisis de la microbiota se realizó una PCR en tiempo real (qPCR), con el objetivo de detectar el ADNr 16S con cebadores específicos para los grupos bacterianos descritos en la Tabla 9. Los experimentos de qPCR se realizaron con un equipo AriaMix (Agilent Technologies, Palo Alto, CA, EE.UU.) utilizando SYBR Green qPCR Master Mix (Agilent Technologies) y siguiendo los programas de amplificación descritos por Redondo y cols. (2019). Se utilizó una curva estándar para cada ensayo de qPCR para la cuantificación del ADN bacteriano diana (**artículo 10**).

Tabla 9. Primers usados en la determinación de la microbiota mediante qPCR.

Organismo	**Secuencia (5' a 3')**	**Tamaño (pb)**	**Tº. anill. (°C)**	**Reference**
Bacteroides spp.	F: ATAGCCTTTCGAAAGRAAGAT R: CCAGTATCAACTGCAATTTTA	495	50ºC	(Matsuki y cols., 2004)
Blautia coccoides	F: AAATGACGGTACCTGACTAA R: CTTTGAGTTTCATTCTTGCGAA	440	50ºC	(Matsuki y cols., 2004)
Clostridium leptum group	F: GCACAAGCAGTGGAGT R: CTTCCTCCGTTTTGTCAA	239	50ºC	(Matsuki y cols., 2004)
Bifidobacterium spp.	F: GATTCTGGCTCAGGATGAACG R: CTGATAGGACGCGACCCCAT	211	60ºC	(Walter y cols., 2001)
Faecalibacterium prausnitzii	F: GATGGCCTCGCGTCCGATTAG R: CCGAAGACCTTCTTCCTCC	199	58ºC	(Bartosch y cols., 2004)
Lactobacillus spp.	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	200	56ºC	(Heilig y cols., 2002)
Enterobacteriaceae	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	195	63ºC	(Bartosch y cols., 2004)
Enterococcus spp.	F: CCCTTATTGTTAGTTGCCATCATT R: ACTCGTTGTACTTCCCATTGT	123	61ºC	(Rinttilä y cols., 2004)

F, forward; pb, pares de bases; R, reverse; Tº. anill., temperatura de anillamiento

3.3.4.11. Determinación de Ácidos Grasos de Cadena Corta

Los niveles de AGCC se determinaron según el método descrito por Álvarez-Cilleros y cols. (2020). Para ello, 0,1g de heces extraídas en condiciones de máxima higiene del colon distal se suspendieron en 1mL de agua con ácido fosfórico al 0,5% y se congelaron a -20ºC. Posteriormente se homogeneizaron en un vórtex durante 2min y se centrifugaron a 17949 *g* durante 10min. La determinación se realizó por

cromatografía de gases Agilent 7890A (Agilent Technologies) equipado con un detector de espectrómetro de masas 5975C y una columna Agilent DB-WAXtr (100% polietilenglicol, 60m, 0,325mm, 0,250µm). El espectrómetro de masas se ajustó durante las mediciones y se utilizó el modo de monitorización de iones únicos para la cuantificación de la señal. Las temperaturas de la fuente de ionización y del cuadrupolo fueron de 230ºC y 150ºC, respectivamente. Se preparó una solución madre con los estándares de interés (WSFA-2; Sigma-Aldrich) y un estándar interno (ácido 4-metilvalérico). Se preparó una curva de calibración de 2 a 10.000µM. Las concentraciones de AGCC se expresaron como µM/g de heces (**artículo 10**).

3.3.4.12. Análisis estadístico

Para el análisis estadístico se utilizó el programa SPSS versión 25 (SPSS Inc., Chicago, Illinois, EE.UU.), mientras que los gráficos fueron elaborados con GraphPad Prism versión 8 (GraphPad software Inc., La Jolla, California, EE.UU.). Los resultados se expresaron como los valores medios con su desviación estándar. Se consideraron diferencias significativas entre grupos con un valor de $p<0,05$.

Se determinó la normalidad de distribución de los datos mediante el test de Kormogorov-Smirnov. Se aplicó el análisis univariado de varianza (ANOVA) para estudiar los efectos entre más de dos grupos, seguido de las comparaciones múltiples mediante la corrección *post-hoc* de Bonferroni para varianzas similares y el de T2-Tamhane para varianzas diferentes. Se utilizó la prueba de Krukal-Wallis seguido del post-hoc de Mann-Whitney para realizar comparaciones múltiples no paramétricas. Las comparaciones entre dos grupos se realizaron mediante el test de la *t* de Student o *U* de Mann-Whitney según la distribución fuera paramétrica o no paramétrica. La distribución de ratas entre los grupos (ej: hipercolesterolémica, clasificación según score, etc) se compararon mediante la prueba de la chi-cuadrado (χ^2). Por último, para evaluar las posibles relaciones lineales entre parámetros de todos los experimentos, se empleó el test de correlación producto-momento de Pearson.

Expresión de resultados

Para una lectura más fácil, el próximo apartado de resultados se ha divido en tres capítulos, que responden a los objetivos específicos planteados en esta Tesis Doctoral. Cada capítulo está divido en subsecciones donde se incluyen las publicaciones correspondientes, junto con una breve introducción, hipótesis del estudio, resultados y conclusiones.

IV. RESULTADOS

Capítulo 1

Estudio a corto plazo de los efectos del extracto de algarroba sobre la digestión y absorción de hidratos de carbono y grasas

Antecedentes: los estudios epidemiológicos sugieren una infinidad de efectos beneficiosos asociados a las dietas ricas en polifenoles, entre los que destaca su acción antioxidante, anticancerígena, antidiabética, antiinflamatoria, neuroprotectora y antimicrobiana (Quiñones y cols., 2013). Concretamente, las PACs se han postulado como un ingrediente funcional prometedor debido a su efecto para modular el metabolismo de los hidratos de carbono y las grasas, destacándose como agente antidiabético (Rauf y cols., 2019). El CFE es una fuente muy rica de este tipo de polifenoles, sin embargo, su posible efecto reductor de la glucemia y lipemia postprandiales nunca ha sido estudiado. Asimismo, los estudios disponibles hasta la fecha, informan de tratamientos prolongados para observar una mejora clara.

Objetivos:

- Evaluar el efecto *in vitro* del extracto de algarroba sobre las enzimas α-glucosidasa (actividad maltasa) y lipasa pancreática, implicadas en la digestión de los hidratos de carbono y las grasas, respectivamente.
- Estudiar y comparar los efectos de la administración aguda y subcrónica (1 semana) de CFE sobre la glucemia postprandial en ratas Wistar macho de dos meses de edad.
- Analizar la modulación nutrigenómica de la administración de CFE sobre los niveles del transportador SGLT1 en duodeno y yeyuno tras el experimento subcrónico.
- Estudiar y comparar los efectos de la administración aguda y subcrónica de CFE sobre la lipemia (trigliceridemia y colesterolemia) postprandial en ratas Wistar macho de dos meses de edad.
- Caracterizar el perfil lipídico del remanente de grasa del lumen intestinal y fecal tras el estudio de digestibilidad en ratas Wistar macho control y con administración de CFE.
- Estudiar aspectos tecnológicos de la formulación de emulsiones simples y gelificadas con CFE añadido.
- Evaluar el efecto de la adición de CFE sobre la lipolisis *in vitro* de las emulsiones y la actividad antioxidante del remanente lipídico.

Publicación 1

Fiber purified extracts of carob fruit decrease carbohydrate absorption

Macho-González A, Garcimartín A, López-Oliva ME, Bertocco G, Naes F, Bastida S, Sánchez-Muniz FJ & Benedí J

Food & Function

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20 de 133 en Food Science & Technology (1er cuartil)

116 de 293 en Biochemistry & Molecular Biology

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Antecedentes: la hiperglucemia postprandial se considera un factor de riesgo para la DMT2 y se ha relacionado con muchas de las complicaciones macro y microvasculares derivadas de esta patología. Las estrategias que se han manejado hasta la fecha para minimizar este estado se han basado en la inclusión de fibra dietética en las comidas, así como la utilización farmacológica de inhibidores de la α-glucosidasa. Actualmente se sabe que los polifenoles pueden mejorar la progresión de la DMT2 al modular el metabolismo de los hidratos de carbono a diferentes niveles. El extracto de algarroba (CFE, por sus siglas en inglés, *carob fruit extract*), rico en fibra insoluble y PACs poliméricas de elevado pero molecular, ha demostrado propiedades hipolipemiantes tanto en estudios en animales como en humanos. Sin embargo, su efecto sobre la hiperglucemia postprandial nunca ha sido probado.

Hipótesis: el extracto de algarroba rico en PACs es capaz de inhibir etapas clave de la digestión de los hidratos de carbono tanto *in vitro* como *in vivo*.

Resultados: concentraciones desde 0,5mg/mL de CFE fueron capaces de inhibir la enzima α-glucosidasa actividad maltasa, alcanzando una inhibición del 50% para la concentración máxima probada de CFE (5mg/mL). Al incrementar la concentración de sustrato, descubrimos que el CFE ejerce un mecanismo de inhibición de tipo competitivo, con una cinética similar a la del fármaco Acarbosa para la mayor dosis de CFE. En un intento de verificar *in vivo* los resultados obtenidos *in vitro*, se realizaron dos estudios de digestibilidad (agudo y subcrónico). El estudio agudo realizado en ratas sanas reveló una reducción de la hiperglucemia postprandial para las diferentes dosis de CFE probadas a la hora de experimento. Tras una semana de administración (estudio subcrónico), el CFE bloqueó la absorción de los hidratos de carbono en mayor medida, reflejando una disminución de la hiperglucemia postprandial del 20% para la menor dosis testada. En la comparativa entre el estudio agudo y subcrónico se observó una clara disminución para la máxima dosis evaluada, resultado que puede deberse a la menor digestión de los hidratos de carbono (inhibición de la α-glucosidasa) y a su menor absorción (descenso en los niveles del transportador de glucosa SGLT1 en duodeno).

Conclusiones: el CFE ejerce importantes efectos en el metabolismo de los hidratos de carbono, reduciendo notablemente la hiperglucemia postprandial desde una primera dosis y mejorando sus efectos tras una semana de consumo, lo que destaca la importancia de la nutrigenómica. Estas propiedades señalan al CFE como una adecuada estrategia nutricional para manejar la hiperglucemia postprandial y reducir las posibles complicaciones de las personas con DMT2.

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Fiber purified extracts of carob fruit decrease carbohydrate absorption

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The postprandial state plays a central role in the development and setting of chronic diseases. Condensed tannins (CT) are polyphenols with a known ability to modify carbohydrate digestion and absorption. The high concentration of CT in the pulp of carob fruit suggests a potential antidiabetic effect. The aim of this work was to analyze the *in vitro* and *in vivo* effects of carob fruit extract (CFE) on the digestion and absorption of carbohydrates. α-Glucosidase activity and glucose diffusion were tested *in vitro* using 0.5, 1, 2 and 5 mg mL^{-1} CFE concentrations. Two *in vivo* absorption studies, acute and subchronic, were carried out in four groups of 6 two-month-old male Wistar rats (control and CFE 25, 50 and 150 mg per kg b.w.), administering 1 mL of olive oil and 0.5 g per kg b.w. of glucose solution by oral gavage. CFE significantly inhibited α-glucosidase activity, through a competitive mechanism, from 1 mg mL^{-1}, and also reduced glucose diffusion in a dose-dependent manner. In the acute study, CFE (50 and 150 mg per kg b.w.) significantly reduced the area under the curve (AUC) of blood glucose. Subchronic CFE administration induced further AUC decreases; and CFE at 150 mg per kg b.w. reduced sodium–glucose-linked transporter-1 (SGLT1) levels in the duodenum. This study demonstrates the hypoglycemic properties of CFE, highlighting its potential role as a suitable nutritional strategy in diabetic patients.

1. Introduction

The typical Western diet, associated with a high intake of total and saturated fat and simple carbohydrates, is causing increasing prevalence of cardiovascular diseases (CDV) and type 2 diabetes mellitus (T2DM).[1] Besides obesity, postprandial hyperglycemia is also considered a decisive risk factor associated with the long-term onset of microvascular and macrovascular complications.[2,3] Therefore, α-glucosidase inhibitors such as acarbose are commonly prescribed to reduce postprandial hyperglycemia.[4] In this regard, patients with abnormal glucose tolerance treated with acarbose present lower progress towards T2DM[4] and show an improvement of insulin sensitivity and CVD markers.[5]

Fiber-rich food consumption has been known to exert beneficial effects in T2DM patients, partially due to their phytochemical content, especially for its anti-inflammatory effect.[6] In fact, polyphenol intake reduces CVD risk[7,8] and exerts a hypoglycemic effect in diabetic rats induced by streptozotocin (STZ).[9] Current evidence indicates that various dietary polyphenols are able to influence the metabolism of carbohydrates at different levels.[10] This response can be partially explained by (1) the reduction of carbohydrate digestion inhibiting pancreatic α-amylase and α-glucosidase activities; (2) limiting glucose absorption at the intestinal level, by partially inhibiting sodium–glucose-linked-transporter-1 (SGLT1) and glucose transporter 2 (GLUT2), major glucose transporters in the small intestine; and (3) modulating diverse pathways involved in carbohydrate metabolism.[8,10] Thus, dietary polyphenols affect postprandial hyperglycemia through a mechanism similar to acarbose, and hence retard the progression to T2DM.[11]

Carob fruit pulp extracts (CFE) (*Ceratonia siliqua* L.) contain large amounts of insoluble polyphenols (65–97% of the product) and small amounts of soluble polyphenols (0–2% of the product).[12] The insoluble fraction is composed of high-molecular-weight polymeric proanthocyanidins, also known as highly condensed tannins (CT). These extracts have demonstrated efficacy in lowering cholesterol without either antinutrient or astringent effects.[13,14] In addition, there are reports that CT from CFE and its active metabolites could exert a systemic effect, producing hypocholesterolemia in both animals and humans.[14,15] However, to the best of our knowl-

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Publicación 2

Effects of Fiber Purified Extract of Carob Fruit on Fat Digestion and Postprandial Lipemia in Healthy Rats

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Antecedentes: la hipertrigliceridemia postprandial se ha reconocido como un evento previo a la instauración de la RI e intolerancia a la glucosa (Aslam y cols., 2016). El control de la hipertrigliceridemia postprandial es uno de los objetivos de la terapia nutricional, tanto de forma preventiva para ralentizar el progreso a DMT2, como tratamiento para reducir las complicaciones diabéticas (Chan y cols., 2013). La fibra y los polifenoles pueden dificultar la absorción de macronutrientes y aumentar la velocidad de tránsito intestinal mediante, lo que conduce a una menor respuesta postprandial. Asimismo, los polifenoles han demostrado regular la homeostasis lipídica a diferentes niveles. El CFE es fuente de estos dos componente y ha revelado ser eficaz para reducir el colesterol plasmático en humanos y ratas, lo que lo predispone como un prometedor agente hipolipemiante (Ruiz-Roso y cols., 2010).

Hipótesis: el CFE rico en fibra y PACs reduce la hiperlipemia postprandial en ratas Wistar sanas de 2 meses de edad.

Resultados: el CFE inhibió la actividad de la lipasa pancreática *in vitro* de forma dosis dependiente, llegando a alcanzar un 20% de inhibición para la máxima concentración probada. Estos resultados fueron corroborados en un estudio de digestibilidad *in vivo*, donde se observó una reducción de la trigliceridemia postprandial en todas las dosis administradas desde una única administración. A su vez, estos resultados fueron más potentes tras una semana administrando el CFE, inhibiendo casi en su totalidad la absorción de triglicéridos en los animales que tomaron la mayor dosis. La misma tendencia fue observada en la colesterolemia postprandial tanto en el estudio agudo como en el subcrónico, lo que afianzó los efectos hipolipemiantes del CFE. La caracterización lipídica del remanente intestinal tras el estudio de digestibilidad justificó nuestra hipótesis al encontrarse un mayor contenido de triglicéridos en el grupo con mayor dosis de CFE, lo que fue indicativo de una clara disminución de la digestión. La mayor presencia de grasa en heces (triglicéridos y colesterol) en este mismo grupo respecto al control, ratificó la menor absorción de lípidos.

Conclusiones: el CFE reduce la hiperlipemia postprandial desde una única dosis y de forma dosis dependiente, inhibiendo la digestión y absorción de grasas a diferentes niveles. Posiblemente estos resultados se deban a los efectos de la fibra y las PACs: a) reduciendo el vaciamiento gástrico, b) inhibiendo la digestión y absorción de grasas, c) incrementando la velocidad de tránsito intestinal, y d) aumentando la síntesis y excreción de sales biliares. Además, el potente efecto hipolipemiante encontrado en el experimento subcrónico *vs.* agudo nos hacen pensar que su consumo induce cambios nutrigenómicos que son necesarios de estudio.

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Effects of Fiber Purified Extract of Carob Fruit on Fat Digestion and Postprandial Lipemia in Healthy Rats

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Supporting Information

ABSTRACT: Increased postprandial lipemia is a cardiovascular disease (CVD) risk factor. Carob fruit extract (CFE) contains condensed tannins, and their intake has been inversely related to CVD. The objective was to evaluate the in vitro pancreatic lipase activity in the presence of CFE and the in vivo effect of CFE on postprandial lipemia of healthy Wistar rats in acute and subchronic digestibility studies and to relate it with changes in fat digestion and absorption. CFE significantly reduced pancreatic lipase activity. A peak delay and a dose-dependent decrease in plasma triglyceride and cholesterol areas under the curve were observed, effects that increased after the subchronic treatment. The levels of nondigested, nonabsorbed triglycerides of the remaining intestinal lumen fat were significantly higher in the maximum dose of CFE administrated versus the control ($P < 0.05$). This study demonstrates for the first time the hypolipemic properties of CFE from the first administration, modifying postprandial lipemia by reducing the extents of fat digestion and absorption.

KEYWORDS: *postprandial triglyceridemia and cholesterolemia, pancreatic lipase, lipemia, fat digestion, condensed tannins, dietary fiber, carob fruit*

INTRODUCTION

Postprandial hypertriglyceridemia (PPHG) is one of the basic pillars of nutritional intervention, because it is closely linked to insulin resistance (IR) and type 2 diabetes mellitus (T2DM) development.[1−3] Therefore, PPHG normalization through nutritional strategies should protect from anti-IR therapy and consequently from the onset of T2DM. Unlike postprandial hyperglycemia, effective therapies against PPHG are not entirely clear,[4] so more information about natural compounds capable of improving PPHG without the need for pharmacological treatment is required.

Dietary fiber and polyphenols are two of the most widely studied compounds used to manage postprandial dysmetabolism.[5] In fact, current epidemiological studies link dietary fiber and polyphenol consumption with protection against cardiovascular disease (CVD) and T2DM.[6,7] Dietary fiber has been related to decreases in the risk of CDV by hindering macronutrient absorption, reducing postprandial responses, and increasing the intestinal transit speed by mechanical mucosa stimulation.[8,9] Associated with dietary fiber, effects on carbohydrate metabolism, especially reducing blood glucose and improving IR,[10] and on fat metabolism by regulating lipid and lipoprotein homeostasis[11,12] have been attributed to polyphenols. With regard to the impact of polyphenols on PPHG, Sugiyama et al.[13] observed an in vitro inhibitory effect of apple polymeric proanthocyanidins on pancreatic lipase. Subsequently, they tested this effect after acute administration in both animals and humans. Likewise, Koo et al.[14] reviewed the effect of green tea procyanidins on lipid metabolism, promoting the reduction of intestinal lipid absorption and/or PPHG.

As a polyphenol source, carob fruit extract (CFE) contains 65% insoluble polyphenols and 2% soluble polyphenols. It is primarily composed of high-molecular weight polymeric proanthocyanidins or condensed tannins (CTs), constituted by numerous flavan-3-ol units.[15,16] CT consumption has been associated with a decrease in the risk of T2DM and CDV.[17,18] Also, CFE has been shown to effectively reduce plasma cholesterol in humans and rats without exerting an antinutrient or astrigent effect.[19,20] Besides, it has also been shown to be effective in improving dyslipidemia and hepatic lipid metabolism in rabbits.[21] A recent study by our group showed that CFE considerably reduced postprandial glycemia after a single administration and enhanced these effects after a week of consumption.[22]

Given the premises mentioned above, our hypothesis is that CFE affects lipid digestion and absorption and reduces PPHG

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Table 1. In Vitro Effect of Carob Fruit Extract (CFE) on Porcine Pancreatic Lipase[a]

	% lipase activity
control	100 ± 0.00 a
1 mg/mL CFE	90.46 ± 2.53 b
2.5 mg/mL CFE	89.20 ± 2.42 b
5 mg/mL CFE	84.30 ± 3.47 c
10 mg/mL CFE	80.52 ± 2.87 c
ANOVA	<0.001

[a]Results are the percentage of pancreatic lipase activity with respect to the control (mean ± SD). Values followed by different letters were significantly different (ANOVA, followed by the T2-Tamhane post hoc test).

in a dose−effect relationship. This study aimed to test the effect of CFE doses on (a) in vitro pancreatic lipase activity, (b) postprandial plasma levels of triglycerides (TGs) and cholesterol, (c) the lipid profile of the remaining intestinal lumen fat, and (d) fecal lipid composition. Differences between the baseline and that after CFE treatment for 1 week in results in vivo were also assessed.

MATERIALS AND METHODS

Carob Fruit Pulp Extract. CFE is a natural insoluble dietary fiber, obtained from the carob pulp following the procedure described in Patent WO2004/014150.[15] Once the seed was removed, a washing step was performed in triplicate runs for 45 min with water at 45 °C while the sample was being constantly stirred, and finally, the washing liquid was discarded. The resulting wet pulp was homogenized with water in a 1:2 ratio (w/w) and vigorously stirred for 3 h at 80 °C. Then, the homogeneous pulp was filtered and the obtained liquid allowed to cool and subsequently subjected to a decanting process at room temperature for 6 h. Finally, the decanted sample was centrifuged at 2500*g* and the supernatant removed. The resulting residue was dried in a heater at 85 °C for 12 h and 0.2 atm of pressure. Once cooled, the residue was powered using a hammer mill and sieved until a size of <180 μm was obtained. According to Bastida et al.[23] and the Patent WO2004/014150, the average composition was as follows: 4.5−7% proteins, 0.5−1% fats, 1.5−3.5% sugars, 3−4% ash (0.6−1.1% calcium, 0.02−0.026% sodium, 0.025−0.047% potassium, and 0.01−0.016% iron), and 74−84% total dietary fiber (with 1−3% soluble fiber and 71−81% insoluble fiber). Moreover, the polyphenols are composed of 34−48% non-extractable condensed tannins and 0.5−1% soluble extractable polyphenols. According to Patent WO2006/000551, the molecular weights of CFE tannins ranged from 6000 to 30000 Da, thus permitting estimation of a range of the degree of polymerization of 26−133 for flavan-3-ol.

In Vitro Pancreatic Lipase Activity. In vitro pancreatic lipase activity was measured following a slight modification of the method of Winker et al.[24] Briefly, 100 μL of the supernatant of porcine pancreatic lipase (10 mg/mL) (Sigma-Aldrich, Madrid, Spain) was mixed with 100 μL of a CFE suspension in phosphate-buffered saline (PBS) and maintained for 10 min at 37 °C. In control samples, the pancreatic lipase solution was mixed with 100 μL of PBS. A fresh substrate solution was prepared with 30 mg of *p*-nitrophenylpalmitate (Sigma-Aldrich), dissolved in 10 mL of isopropanol, and mixed with 90 mL of PBS (pH 8.0) containing 207 mg of sodium deoxycholate and 100 mg of gum arabic (Sigma-Aldrich); 1.6 mL of this substrate solution preheated to 37 °C was added to pancreatic lipase and CFE. The reaction mixture was kept at 37 °C while being constantly stirred for 20 min and then introduced into ice for 5 min to stop the reaction. Lipase activity was measured spectrophotometrically at 410 nm (SPECTROstar Nano, BMG LABTECH, Offenburg, Germany). All tests were performed in triplicate, and the pancreatic lipase activity was measured twice in each sample. Data were expressed as the percentage of activity with respect to the control.

Experimental Design for Digestibility Studies. Twenty-four male Wistar rats were divided into four groups of six rats each: control and CFE 25, CFE 50, and CFE 150 mg/kg bw based on the dose of CFE in milligrams per kilogram of body weight (bw) administrated daily by oral gavage.[22] The control group received deionized water. Rats were treated for a week, and digestibility studies (acute and subchronic) were performed on day 1 and day 7, respectively. During this period, animals consumed a chow diet (Global Diet 2014, Panlab, Barcelona, Spain) ad libitum. Both digestibility studies were performed under overnight-fasting conditions. Rats received by oral gavage 1 mL of a D-glucose solution (0.5 g/mL) and 1 mL of olive oil carrying in suspension the corresponding dose of CFE. Blood (approximately 125 μL) was collected hourly in a cool heparinized

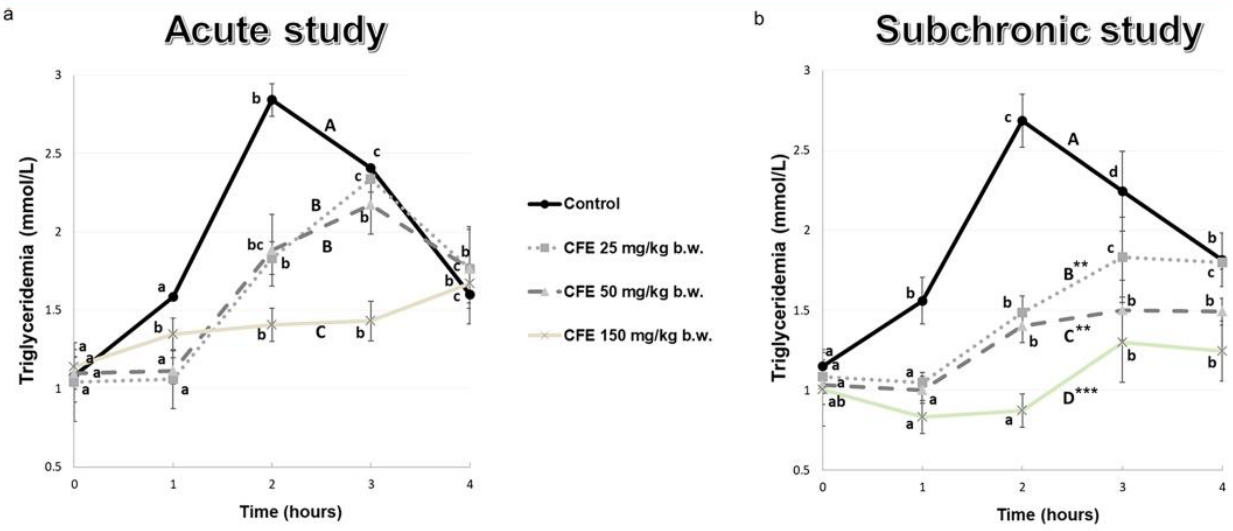

Figure 1. Effects of carob fruit extract (CFE) on postprandial triglyceridemia: (a) acute study and (b) subchronic study. Values are means ± SD ($n = 6$). Postprandial triglyceride time evolution (0 vs 1, 2, 3, or 4 h) values in a given group bearing different lowercase letters were significantly different ($P < 0.001$). Lines showing the postprandial triglyceridemia trend for the same study length bearing different uppercase letter were significantly different ($P < 0.001$; repeated measures followed by a Bonferroni post hoc test). Lines showing the postprandial triglyceridemia trend for the same dose bearing asterisks (**$P < 0.01$; ***$P < 0.001$; repeated measures) were significantly different.

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

tube from the tail for a period of 4 h. At the end of the subchronic postprandial study (after treatment for 4 h), rats were anesthetized with isoflurane (5%) and euthanized by extracting blood from the descending aorta with a heparinized syringe. The small intestine was washed with 10 mL of a 0.9% saline solution, collecting the remaining intestinal content using the method described by Sánchez-Muniz et al.[25] and preserving it at −80 °C until analysis could be performed. Feces were collected the first and last day of the experiment and dried at 100 °C in an oven for 3 days. The study was approved by the Spanish Science and Technology Advisory Committee (Project AGL2014-53207-C2-2-R) and by an Ethics Committee of the University of Alcalá (CEI-UAH-AN-2014010). All experiments were performed in compliance with Directive 86/609/EEC of November 24, 1986 (amended by Directive 2003/65/EEC of July 22, 2003) for the protection of animals used for experimentation.

Determination of Plasma Triglyceride and Cholesterol Levels. Plasma was obtained after the centrifugation of blood at 986*g* for 10 min. Postprandial TG and cholesterol were quantified in a plate reader (SPECTROstar Nano), at 492 nm, using the Triglycerides-LQ and Cholesterol-LQ kits (Spinreact, Barcelona, Spain), respectively, according to the manufacturer's manual. In addition, the 4 h area under the curve (AUC) was calculated from TG and cholesterol plasma concentrations.

Remaining Intestinal Lumen Fat Content and Composition. Fat from the remaining intestinal lumen content was extracted after a 4 h oil gavage with a chloroform/methanol mixture according to the method described by Sánchez-Muniz.[25] High-pressure size exclusion chromatography (HPSEC) was performed to elucidate its lipid composition as described by Dobarganes et al.[26] Fifteen milligrams of extracted fat diluted in 1 mL of tetrahydrofuran was applied in a high-performance liquid chromatograph (1100 series, Agilent Technologies, Madrid, Spain) with a 20 μL sample loop. A refractive index detector (1260 infinity, Agilent Technologies) and two serially connected 300 mm × 7.5 mm (inside diameter) (5 μm particle size), 0.01 and 0.05 μm, PL gel columns (Agilent, Bellefonte, PA) were operated at 40 °C. High-performance liquid chromatography grade tetrahydrofuran was used as the mobile phase with a flow rate of 1 mL/min. Nondigested, nonabsorbed TG, diglycerides (DG), monoglycerides (MG), and free fatty acids (FFA) were quantified as grams per 100 g of sample.

Fecal Fat Extraction. Fecal fat was extracted according to the method described by Olivero-David et al.[27] One gram of dried feces was hydrated with 2 mL of water for 12 h at 4 °C. Subsequently, it was homogenized in ultraturrax T25 (IKA) until a homogeneous solution was obtained. Four milliliters of a chloroform/methanol mixture (1:1) was added and the mixture homogenized again with ultraturrax T25 (IKA). Samples were centrifuged for 10 min at 750*g*, and the chloroform phase was collected. This process was repeated three times. Afterward, the solvents were evaporated with a rotary evaporator (Rotavapor R-200, Büchi, Barcelona, Spain). The extracted fat was solubilized with 1 mL of *tert*-butanol (Sigma-Aldrich), vigorously stirred for 15 min at 37 °C, and centrifuged at 9850*g* for 5 min, and the supernatant obtained was evaluated with the TG and cholesterol kits (Spinreact) mentioned above.

Statistical Analysis. Statistics were determined using SPSS version 25.0 (SPSS Inc., Chicago, IL). Results are expressed as means ± the standard deviation (SD). The Kolmogorov−Smirnov test was used to analyze the normal distribution of data. Time evolution of postprandial lipemia (cholesterol and TG) was tested using the General Linear Model (GLM) of repeated measures test followed by the Bonferroni post hoc test according to the doses (control and CFE 25, CFE 50, and CFE 150 mg/kg bw) and study length (acute and subchonic) factors and interactions. Postprandial TG and cholesterol at the different study hours, TG and cholesterol AUC analysis, and fecal fat composition were tested by the GLM univariate procedure taking into account two fixed factors, CFE doses and study length, followed by the T2-Tamhane or Bonferroni post hoc test after assuming inequality or equality of variances (squared SD), respectively. One-way analysis of variance (ANOVA) followed by the T2-Tamhane or Bonferroni post hoc test was applied to

Table 2. Effect of Carob Fruit Extract (CFE) on Postprandial Plasma Triglyceride Levels (millimoles per liter) in Acute and Subchronic Studies[a]

						ANOVA		
study hour	study length	control	CFE 25 mg/kg bw	CFE 50 mg/kg bw	CFE 150 mg/kg bw	dose effect	study length effect	dose−study length interaction
0	acute	1.09 ± 0.17	1.05 ± 0.25	1.11 ± 0.10	1.13 ± 0.06	nonsignificant	nonsignificant	nonsignificant
	subchronic	1.15 ± 0.10	1.09 ± 0.10	1.05 ± 0.13	1.00 ± 0.23			
1	acute	1.59 ± 0.02 a	1.07 ± 0.19 b	1.11 ± 0.08 b	1.34 ± 0.10 b	<0.001	<0.001	<0.001
	subchronic	1.57 ± 0.15 a	1.05 ± 0.06 b	1.00 ± 0.08 bc*	0.84 ± 0.10 c***			
2	acute	2.84 ± 0.10 a	1.84 ± 0.10 b	1.88 ± 0.23 b	1.40 ± 0.10 c	<0.001	<0.001	0.008
	subchronic	2.70 ± 0.17 a	1.48 ± 0.10 b***	1.40 ± 0.10 b***	0.88 ± 0.10 c***			
3	acute	2.40 ± 0.02 a	2.34 ± 0.08 ab	2.17 ± 0.19 b	1.42 ± 0.13 c	<0.001	<0.001	0.002
	subchronic	2.24 ± 0.25 a	1.84 ± 0.25 b***	1.50 ± 0.19 bc***	1.30 ± 0.25 c			
4	acute	1.61 ± 0.19	1.76 ± 0.25	1.76 ± 0.27	1.67 ± 0.13	<0.001	0.037	<0.001
	subchronic	1.82 ± 0.17 a	1.80 ± 0.04 a	1.48 ± 0.08 b	1.25 ± 0.19 c***			
total triglyceride AUC (mmol L^{-1} min^{-1})	acute	509.65 ± 10.72 a	398.15 ± 6.92 b	395.53 ± 14.38 b	335.13 ± 17.20 c	<0.001	<0.001	<0.001
	subchronic	478.71 ± 22.05 a	348.3 ± 23.24 b**	310.37 ± 20.57 b***	248.29 ± 27.13 c***			

[a]Results are plasma triglyceride means ± SD (n = 6). Values in the same row (same study length and different dose) bearing different letters were significantly different. Values in the same column and study hour (acute vs subchronic study) bearing asterisks were significantly different (*P < 0.05; **P < 0.01; ***P < 0.001).

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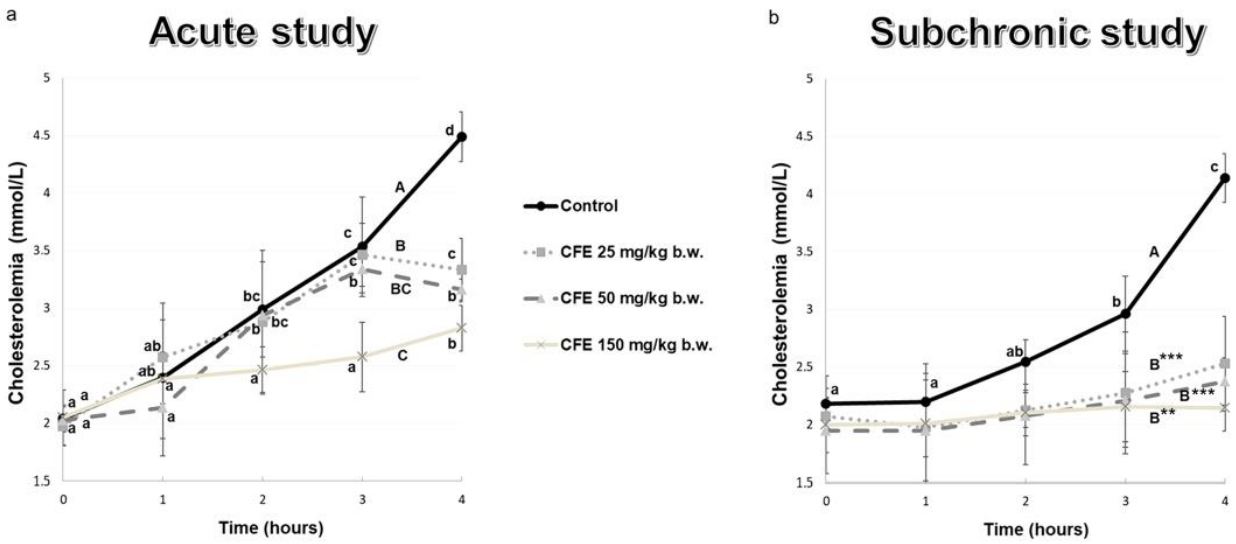

Figure 2. Effects of carob fruit extract (CFE) on postprandial cholesterolemia: (a) acute study and (b) subchronic study. Values are means ± SD (n = 6). Postprandial cholesterol time evolution (0 vs 1, 2, 3, or 4 h) values in a given group bearing different lowercase letters were significantly different (P < 0.05). Lines showing the postprandial cholesterolemia trend for the same study length bearing different uppercase letter were significantly different (P < 0.001; repeated measures followed by the Bonferroni post hoc test). Lines showing the postprandial cholesterolemia trend for the same dose bearing asterisks (**P < 0.01; ***P < 0.001; repeated measures) were significantly different.

compare in vitro pancreatic lipase activity or the dose effect on the remaining lumen fat content, respectively. Differences were considered significant at the P < 0.05 level.

RESULTS

In Vitro Pancreatic Lipase Activity. Table 1 summarizes the effect of CFE on in vitro pancreatic lipase activity. CFE significantly reduced the activity of pancreatic lipase from the lowest concentration tested (1 mg/mL), acting in a dose-dependent manner with a maximum inhibition of 19.48% for 10 mg/mL CFE.

Effect of CFE on Plasma Triglyceride Levels in the Postprandial Studies. The postprandial triglyceridemia curves over 4 h during the acute and subchronic studies are shown in panels a and b of Figure 1, respectively. A significant interaction between study hour and study length was observed (P < 0.001). CFE treatments (25, 50, and 150 mg/kg bw) significantly reduced the PPHG compared to the control (all P < 0.001) in study length with a low level of PPHG in the subchronic study (P < 0.001). In the acute study, CFE 25 and 50 mg/kg bw did not reflect the increase in the level of TG during the first hour, revealing the maximum TG peak at 3 h for all CFE groups and at 2 h for the control group (Figure 1a). CFE 150 mg/kg bw did not show an increase in the level of postprandial TG until 3 h, in contrast to CFE 25 and CFE 50 mg/kg bw, which showed increases at 2 h, or the control from the first hour of study (Figure 1b).

Table 2 shows the effect of the dose and study length for each analyzed hour and for total TG AUC. Significant dose−study length interactions (at least P = 0.008) were observed on plasma TG from the first hour to the fourth hour and on TG AUC (P < 0.001). CFE dose and study length significantly affected postprandial plasma TG values from the first hour to the fourth (at least P = 0.037). CFE, in a dose-dependent manner, tended to reduce the plasma TG level with respect to the control. Significant differences were observed between the acute and subchronic studies at 2 and 3 h in the CFE 25 mg/kg bw group, at 1, 2, and 3 h in the CFE 50 mg/kg bw group, and at 1, 2, and 4 h in the CFE 150 mg/kg bw group (P < 0.001). The total TG AUC differed among groups (CFE 150 mg/kg bw < CFE 50 and CFE 25 mg/kg bw < control; P < 0.001). Nonsignificant differences (P > 0.05) were observed for the control group between the acute and subchronic studies. In the three treatment groups, CFE induced a significant reduction in TG AUC in the subchronic study with respect to the acute one (at least P = 0.001) (Table 2).

Effect of CFE on Plasma Cholesterol Levels in the Postprandial Studies. The postprandial cholesterolemia curves over 4 h during the acute and subchronic studies are shown in panels a and b of Figure 2, respectively. A nonsignificant interaction between study hour and study length was observed (P > 0.7). CFE treatments (25, 50, and 150 mg/kg bw) significantly reduced the postprandial cholesterolemia compared to the control (all P < 0.001) in both study length with lower cholesterolemia in the subchronic study (P < 0.001). In the acute study, the plasma cholesterol level of increased from the second hour (P < 0.05) in the control and CFE 25 and CFE 50 mg/kg bw groups. The plasma cholesterol level increased in the control from the 3 h point (P < 0.05) but did not change in any CFE treatment throughout the subchronic study.

A significant dose−study length interaction (P = 0.020) was observed for plasma cholesterol at the 4 h point (Table 3). CFE dose and study length significantly affected postprandial plasma cholesterol values from the second hour (at least P = 0.032). CFE, in a dose-dependent manner, tended to reduce plasma cholesterol levels with respect to the control. Significant differences were observed between the acute and subchronic studies at 2, 3, and 4 h in all CFE treatment groups (P < 0.05). Nonsignificant differences (P > 0.05) for the control group cholesterol AUC between the acute and subchronic studies were observed. In the three treatment groups, CFE induced a significant reduction (at least P < 0.05) in the cholesterol AUC in the subchronic study with respect to the acute counterpart (Table 3).

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 3. Effect of Carob Fruit Extract (CFE) on Postprandial Plasma Cholesterol Levels (millimoles per liter) in Acute and Subchronic Studies[a]

						ANOVA		
study hour	study length	control	CFE 25 mg/kg bw	CFE 50 mg/kg bw	CFE 150 mg/kg bw	dose effect	study length effect	dose–study length interaction
0	acute	2.04 ± 0.12	1.97 ± 0.17	2.03 ± 0.03	2.05 ± 0.24	nonsignificant	nonsignificant	nonsignificant
	subchronic	2.18 ± 0.22	2.07 ± 0.16	1.95 ± 0.37	2.00 ± 0.17			
1	acute	2.39 ± 0.22	2.57 ± 0.47	2.14 ± 0.42	2.38 ± 0.52	nonsignificant	0.013	nonsignificant
	subchronic	2.20 ± 0.24	1.98 ± 0.26*	1.95 ± 0.50	2.01 ± 0.25			
2	acute	2.99 ± 0.41	2.88 ± 0.63	2.94 ± 0.08	2.46 ± 0.20	0.032	<0.001	nonsignificant
	subchronic	2.55 ± 0.19 a	2.13 ± 0.15 b*	2.08 ± 0.43 ab**	2.10 ± 0.15 b*			
3	acute	3.53 ± 0.43 a	3.46 ± 0.27 a	3.34 ± 0.21 a	2.58 ± 0.30 b	<0.001	<0.001	nonsignificant
	subchronic	2.96 ± 0.33 a	2.27 ± 0.53 ab**	2.21 ± 0.40 b**	2.11 ± 0.08 ab*			
4	acute	4.49 ± 0.22 a	3.33 ± 0.28 b	3.16 ± 0.09 bc	2.83 ± 0.20 c	<0.001	<0.001	0.020
	subchronic	4.14 ± 0.21 a*	2.34 ± 0.41 b**	2.38 ± 0.21 b***	2.14 ± 0.21 b***			
total cholesterol AUC (mmol L^{-1} min^{-1})	acute	709.6 ± 48.0 a	649.3 ± 69.0 ab	619.6 ± 68.8 ab	571.1 ± 64.7 b	<0.001	<0.001	nonsignificant
	subchronic	652.0 ± 42.5 a	503.7 ± 51.7 b**	479.8 ± 66.5 b*	438.8 ± 53.3 b**			

[a]Results are plasma cholesterol means ± SD (n = 6). Values in the same row (same study length and different dose) bearing different letters were significantly different. Values in the same column and study hour (acute vs subchronic study) bearing asterisks were significantly different (*P < 0.05; **P < 0.01; ***P < 0.001).

Quantification and Characterization of the Remaining Intestinal Lumen Fat. Table 4 shows the remaining intestinal lumen fat content and its composition analyzed by HPSEC (Supporting Information). The remaining intestinal lumen fat amount was significantly larger in the CFE 150 mg/kg bw rats than in the other groups (P < 0.05). A greater TG content was observed in CFE 150 mg/kg bw animals than in the control. The CFE 150 mg/kg bw group displayed an amount of absorbable fraction (MG and FFA) significantly larger than that of the control (P = 0.049).

Fecal Moisture, Triglyceride, and Cholesterol Contents. Table 5 shows the results of moisture, TG, and cholesterol in feces. Significant dose−study length interactions for fecal parameters were observed (at least P = 0.001). Both CFE dose and study length significantly affected fecal composition (at least P = 0.003). Nonsignificant differences (P > 0.05) were found in fecal compounds among groups in the acute study, but values for fecal moisture and TG in the CFE 150 mg/kg bw group and fecal cholesterol in all CFE groups were significantly higher than the control values (P < 0.001). Higher fecal moisture, TG, and cholesterol levels (77.7, 46.7, and 24.7%, respectively; all P < 0.001) were found in CFE 150 mg/kg bw rats of the subchronic study than in those of the acute study.

DISCUSSION

Taking into account the previous relevant effects of CFE on postprandial glycemia,[22] this study aimed to evaluate the potential effects of CFE on postprandial triglyceridemia and cholesterolemia through the modification of fat digestion and absorption. Our results demonstrate that CFE reduces postprandial lipemia levels. It is well-known that a lower level of dietary fat absorption may improve PPHG associated with IR and T2DM, along with other cardiovascular risk factors.[1,28] In this regard, our results show for the first time the ability of CFE to inhibit in vitro pancreatic lipase and reduce fat digestion and absorption in healthy rats after an oral overload of 1 mL of olive oil, decreasing PPHG while considerably increasing fecal fat excretion after a 1 week treatment. Thus, CFE should be considered as an appropriate alternative for managing during short-term treatments both PPHG and postprandial hyperglycemia.

Soluble fiber is commonly considered an effective strategy for reducing plasma lipid levels and is usually used in hypercholesterolemic patients[29,30] for bile acid sequestration. Although there is controversy about the effect of insoluble fiber on lipemia, it is being increasingly recommended as it seems to lower TG, total cholesterol, and LDL cholesterol levels.[31,32] Such effects of insoluble fiber may be associated with the fiber itself and/or some of its minor compounds such as polyphenols.[33,34] Again, the large amount of insoluble fiber (cellulose and hemicellulose) and polyphenols present in CFE could be responsible for the hypotriglyceridemic effect found in this experiment. Indeed, in our previous study about the action of CFE on the enzymes involved in carbohydrate digestion, we highlighted the important role of polyphenols.[22]

Observing acute study results and taking into account the fact that only a direct effect can be considered because it is the first administration, we found three main mechanisms seem to be involved in the hypotriglyceridemic effect: (a) delayed gastric emptying, (b) partial blocking of fat digestion and absorption, and (c) increasing the intestinal transit speed. The lower levels of TG in the first hour and especially the delay in

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 4. Effect of Carob Fruit Extract (CFE) on the Amount and Composition of the Remaining Intestinal Lumen Fat[a]

	intestinal fat (mg)	TG (mg/total weight)	DG (mg/total weight)	MG (mg/total weight)	FFA (mg/total weight)	absorbable fraction[b]
control	75.45 ± 5.83 a	4.10 ± 1.32 a	11.58 ± 1.64	15.28 ± 1.47	43.35 ± 2.89	58.63 ± 3.04 a
CFE 25 mg/kg bw	77.11 ± 7.21 a	4.89 ± 1.59 ab	13.51 ± 3.43	18.41 ± 3.37	46.94 ± 3.00	65.35 ± 6.21 ab
CFE 50 mg/kg bw	78.10 ± 8.05 a	6.15 ± 2.21 ab	11.89 ± 2.49	17.48 ± 2.46	43.36 ± 5.52	60.83 ± 5.66 ab
CFE 150 mg/kg bw	88.08 ± 5.37 b	7.13 ± 1.15 b	13.17 ± 1.87	19.43 ± 2.60	49.90 ± 5.52	69.65 ± 8.23 b
P	0.021	0.043	nonsignificant	nonsignificant	nonsignificant	0.049

[a]Values [means ± SD (n = 6)] in the same column bearing different letters were significantly different (ANOVA, followed by the Bonferroni post hoc test). [b]Absorbable fraction being MG and FFA.

Table 5. Effect of Carob Fruit Extract (CFE) on Fecal Moisture, Triglyceride, and Cholesterol Composition[a]

					ANOVA		
	control	CFE 25 mg/kg bw	CFE 50 mg/kg bw	CFE 150 mg/kg bw	dose effect	study length effect	dose–study length interaction
fecal moisture (%)							
acute	17.15 ± 1.66	16.05 ± 0.51	17.46 ± 0.33	16.32 ± 1.55	0.003	<0.001	0.001
subchronic	19.75 ± 2.37 a	24.06 ± 0.96 a**	24.71 ± 2.70 a**	29.00 ± 0.37 b***			
% increase	15.2	49.9	41.5	77.7			
fecal triglycerides [mg (g of dry matter)$^{-1}$ day^{-1}]							
acute	11.60 ± 0.23	11.29 ± 0.38	11.72 ± 1.05	10.74 ± 0.25	0.001	<0.001	<0.001
subchronic	11.65 ± 0.14 a	12.04 ± 0.22 a	12.54 ± 0.96 a	15.69 ± 0.28 b***			
% increase	0.45	6.2	6.8	46.7			
fecal cholesterol [mg (g of dry matter)$^{-1}$ day^{-1}]							
acute	7.75 ± 0.06	7.75 ± 0.07	7.84 ± 0.07	7.69 ± 0.07	<0.001	<0.001	<0.001
subchronic	7.73 ± 0.25 a	9.39 ± 0.06 b***	8.76 ± 0.09 c***	9.57 ± 0.32 b***			
% increase	−0.21	22.1	10.3	24.7			

[a]Values [means ± SD (n = 6)] in the same row bearing different lowercase letters were significantly different. Values for the acute and subchronic study for the same group bearing asterisks were significantly different (*P < 0.05; **P < 0.01; ***P < 0.001).

the peak level from the second to the third hour for all CFE groups with respect to the control clearly account for the effect on gastric emptying. The prolonged residence of fat in the stomach could be related to an increase in viscosity induced by the insoluble fiber,[35] producing a delay in fat gastric emptying and, therefore, in TG digestion and absorption. Our results agree with those of Cires et al.,[36] who posit a link between CT and changes in gastric emptying. Likewise, Serrano et al.[37] also found a decrease in the rate of gastric emptying in Wistar rats fed a proanthocyanidin extract from grape. These authors relate this effect to a gastric motility decrease associated with the increase in the level of GLP-1. In addition, oil viscosity together with the CFE insoluble fiber content could favor the emptying delay,[35] explaining, at least partially, the results presented here. Once more, the fact that the TG AUC was significantly smaller in CFE animals than in their control counterparts suggests that modifications in fat digestion and absorption could also be implicated. In this regard, these results on pancreatic lipase inhibition agree with those of other authors that reported that proanthocyanidins inhibit pancreatic lipase, a key enzyme in fat digestion.[13,36,38] In addition to polyphenol, CFE would also affect digestion, as insoluble fiber is known to reduce the joint action of pancreatic lipase and bile salt on lipid droplets.[39] The hypothesis that CFE affects lipid digestion and absorption in a dose–effect relationship has been demonstrated in our study, which confirms the negative relationship between the AUC for PPHG and both fecal fat and TG levels. Wong et al.[38] and Sugiyama et al.,[13] working on in vitro and in vivo models, suggest that the higher the degree of polymerization of proanthocyanidins, the greater the inhibitory effect. In addition to the effects on gastric emptying and fat digestion and absorption discussed, CFE could exert a third effect, possibly also increasing the intestinal transit speed observed in other fiber studies.[39] Our results are agree with those of Bensalah et al.,[40] who observed a reduction of lipemia in Wistar rats fed a diet rich in insoluble fiber, mainly slow absorption by trapping nutrients, digestive enzymes, or bile acids in the matrix and by slowing mixing and diffusion in the intestine. This, in turn, would give rise to a lower digestion level by increasing the rate of transit through the gastrointestinal tract,[41] as has been demonstrated by testing other insoluble fibers.[39]

The subchronic study again confirmed the CFE hypotriglyceridemic effect observed in the acute study. Nonetheless, TG and cholesterol AUC were significantly lower than in the acute study with the three CFE doses tested. At present, we do not have a clear hypothesis, but treatment length would affect the three effects on AUC already discussed. A stronger effect on fat digestive enzymes and absorption, possibly entailing changes in gene expression and variations in protein levels by the action of diet polyphenols, has been postulated.[11] With regard to the hypothetical mechanism whereby CFE modifies fat digestion, HPSEC analysis of the remaining intestinal lumen fat in the postprandial study revealed a significant increase in the levels of total fat and TG in the CFE 150 mg/kg bw group compared to the control. This would confirm the interference of pancreatic lipase by CFE noted above.[13,14] In addition, CFE decreases the plasma cholesterol level from an acute dose and increases its effect in the subchronic study. Ruiz-Roso et al.[20] demonstrated a decrease in cholesterolemia in hypercholesterolemic subjects after they had consumed a CT-enriched product for 4 weeks. This could be due to the effect of some polyphenols on the metabolism of lipoproteins, especially VLDL formation, as

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

observed by Bansode et al. for procyanidins in postprandial cholesterolemia[42] and by Pérez-Jiménez et al. for a grape extract rich in anthocyanidins and proanthocyanidins.[32] However, the effect of insoluble fiber present in CFE on fat digestion and absorption cannot be ruled out. Bensalah et al. have shown that cellulose hinders the enzyme−substrate interaction, sequesters bile acids, and increases the intestinal transit speed.[40] Thus, the joint action of CFE polyphenols and dietary fiber may produce a decrease in the level of lipid digestion and absorption, as shown in the larger amount of MG and FFA present in the CFE 150 mg/kg bw group compared to the control group, thus justifying the results presented here.

The results of fecal composition were also consistent with the mechanisms proposed above. Thus, the fact that the level of moisture found in the feces of CFE 150 mg/kg bw rats was significantly greater could be due to the differences in bolus characteristics, which would promote faster colonic transit.[43,44] The same animals also showed larger amounts of TG in feces, not only versus the control but also versus the amounts recorded at the beginning of the study. This shows an increase of 36%, reflecting the involvement of mechanisms that reduce the level of fat digestion. All CFE doses significantly increased the fecal cholesterol content versus the control after treatment for 1 week. This could be associated with an impact on bile metabolism, as suggested for persimmon CTs.[45] The poor availability of bile micelles would cause a decrease in PPHG as a result of an increased rate of elimination of fat from feces.[46,47] The effects on bile acids were not tested and will be in future studies.

In summary, this study demonstrates that CFE by a dose-dependent manner reduces PPHG and affects fat digestion and absorption. The effect on PPHG was evident from the first administration, supporting the idea of using CFE as a functional ingredient to reduce postprandial lipemia in subjects at increased risk of IR. More studies are necessary, but these results, added to those of our previous work,[22] lead us to consider the inclusion of CFE in diet therapy for people who need to control the postprandial state.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b01476.

Results showing the largest amounts of triglycerides present in the remaining intestinal lumen fat in the CFE groups in relation to the control, analyzed by HPSEC (PDF)

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F.J.S.-M. collaborated in the design, discussion, and writing of the manuscript. A.M.-G., A.G., M.E.L.-O. and F.N. conducted the experiments, participated in data analysis, and drafted the manuscript. S.B. and J.B. participated in the study design and manuscript review. A.A.-A. and M.J.G.-M. provided technical assistance. All authors read and approved the final manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AUC, area under the curve; CVD, cardiovascular disease; CFE, carob fruit extract; CTs, condensed tannins; DG, diglyceride; FFA, free fatty acid; GLM, general linear model; HPSEC, high-pressure size exclusion chromatography; IR, insulin resistance; PPHG, postprandial hypertriglyceridemia; TG, triglyceride; T2DM, type 2 diabetes mellitus

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Publicación 3

The Effect of Emulsifying Protein and Addition of Condensed Tannins on n-3 PUFA Enriched Emulsionsfor Functional Foods

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Antecedentes: En los últimos años se ha propuesto el uso de emulsiones simples y gelificadas como un sistema tecnológico adecuado para reemplazar grasas saturadas por otras con un perfil más saludable en los productos cárnicos (Jiménez-Colmenero, 2007). El tipo de sistema a elaborar (emulsión simple o gelificada) y la proteína emulsificante utilizada (caseinato sódico, SC; aislado de proteína de suero de la leche, WPI) determinan las propiedades reológicas del nuevo producto. Estos factores condicionan notablemente la digestión de los lípidos, ya que modifican el tamaño de gota y por tanto el acceso de la lipasa (Chang & McClements, 2016; Wilde & Chu, 2011). Por ello, el objetivo del presente trabajo fue evaluar las propiedades reológicas de los productos formulados, así como el efecto de la adición del CFE sobre la oxidación de los sistemas y la lipolisis que acontece tras una digestión *in vitro*.

Hipótesis: El tipo de proteína emulsificante (SC y WPI) determina las propiedades reológicas de las emulsiones simples y gelificadas, al mismo tiempo que modifica la liberación del CFE.

Resultados: El estudio reológico reveló diferentes comportamientos en función de la proteína emulsificante, tanto en las emulsiones simples como gelificadas. La adición de CFE produjo un refuerzo estructural de las emulsiones gelificadas, especialmente en el caso de SC. El resultante obtenido tras la digestión *in vitro* de las diferentes emulsiones mostró una mayor liberación de CFE en las emulsiones simples *vs*. gelificadas, lo que se correspondió con una mayor actividad antioxidante. El análisis lipídico de los productos digeridos reflejó un mayor lipolisis en la emulsión gelificada con WPI que en la emulsión la simple. Además, también reveló una mayor presencia de triglicéridos en los sistemas con CFE añadido en los dos tipos de emulsiones, resultados que no se encontraron en los sistemas formulados con SC.

Conclusiones: La proteína emulsionante y/o la adición de CFE modifican significativamente la estructura de los sistemas, la liberación del compuesto bioactivo, la actividad antioxidante y la digestión de lípidos. La emulsión gelificada con WPI permitió una mayor liberación de lípidos. Sin embargo, es importante estudiar el tipo de sistema, ya que el efecto inhibitorio de la lipasa pancreática por parte del CFE está condicionado por la proteína emulsificante utilizada.

foods

MDPI

Article

The Effect of Emulsifying Protein and Addition of Condensed Tannins on n-3 PUFA Enriched Emulsions for Functional Foods

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Abstract: This paper examines the effect of the type of the emulsifying protein (EP) (sodium caseinate (SC) and whey protein isolate (WPI)) on both oil-in-water liquid-like emulsions (Es) and the corresponding cold gelled emulsions (GEs), and also the effect of addition of carob extract rich in condensed tannins (T). The systems, intended as functional food ingredients, were studied in various different respects, including rheological behaviour, in vitro gastrointestinal digestion with determination of the release of non-extractable proanthocyanidins (NEPA) from T, antioxidant activity and lipolysis. EP significantly affects the rheological behaviour of both Es and GEs. T incorporation produced a structural reinforcement of GEs, especially in the case of SC. The digests from Es displayed a higher antioxidant activity than those from GEs. T lipase inhibition was observed only in the formulations with WPI. Our results highlight the importance, in the design of functional foods, of analyzing different variables when incorporating a bioactive compound into a food or emulsion in order to select the better combination for the desired objective, owing to the complex interplay of the various components.

Keywords: gelled emulsion; sodium caseinate; whey protein isolate; condensed tannins; rheology; lipid digestibility

1. Introduction

In recent years, the use of structured liquid oils from different origins in the form of emulsion gels has been proposed as a novel technological approach to replace saturated fats in order to improve the quality of different foods (meat products, spread products, bakery products, etc.). In this sense, oils are structured to create a plastic fat, which retains solid-like properties while possessing a healthier fatty acid profile [1].

Solid-like emulsions are generated from stable liquid-like oil-in-water emulsions (Es) by gelling the continuous phase and/or aggregating the emulsion droplets. Generally, the gelation process involves thermal, enzymatic or chemical treatments, but recently the use of cold gelling agents as

polysaccharides, proteins and their combination with other ingredients has been proposed. They are able to form a continuous network through polymer interactions, which are responsible for the functional properties of structured emulsion gels [2,3]. These gelled emulsions (GEs) can also act as a vehicle for several bioactive compounds that are different from healthy fatty acids. It was also reported that properties of GEs depend on the nature and complex interactions among their structural components [4].

In this regard, Pintado et al. [3] determined the technological and structural characteristics of GEs formulated with olive oil and chia flour and seeds as emulsifiers, and a cold gelling agent (microbial transglutaminase (MTG), alginate or gelatin), obtaining different relationships between structural properties and textural behavior. These same authors underlined the importance of understanding the complex interactions among the different components of GEs through textural and structural methods to facilitate their incorporation into a suitable food matrix to replace fats in healthy products. Therefore, an improved understanding of the role of each GE component and their interaction would help to develop strategies to modify or design new healthy products. In addition, the composition and structure of the GE systems may play an important role in oil digestion activity by modifying lipid hydrolysis. In this sense, recent studies have suggested that lipid digestion may be potentially influenced by different factors: (a) the nature of the emulsifier/stabilizer used to form emulsions, which affects interfacial phenomenon, (b) oil droplet size, which has an effect on the surface area available for lipase adsorption, and (c) consistency of the systems, which affects mass transport [5,6].

Thus, it would be interesting to design GEs elaborated with different emulsifying proteins (EPs) and a cold gelling system (a mixture of gelatin, κ-carrageenan and MTG) together with a healthy lipid combination (olive, linseed and fish oils), which is designed with polyunsaturated fatty acids (PUFAs) in suitable proportions for purposes of achieving recommended intake objectives [7]. Natural biopolymer molecules (proteins and polysaccharides) can prevent droplet aggregation and stabilize emulsions by forming a thin layer adsorbed on the oil-in-water surface, therefore reducing interfacial tension [8]. Thus, they are extensively used as functional ingredients in the food industry to stabilize emulsions and foams, control the texture and structure of foods, and protect and deliver bioactive ingredients to targeted sites [9]. Proteins are complex macromolecules with different levels of structure that form an immobile viscoelastic adsorbed layer on the surfaces of oil droplets, which mechanically prevent coalescence [5]. Among the proteins that are most widely used as emulsifiers of food-related emulsions are sodium caseinate (SC) and whey protein isolate (WPI) from milk, due to their excellent functional and nutritional properties [4]. However, to the best of our knowledge, there is scarce information in the scientific literature about their role in structured emulsions, including their implication on digestion. In this context, it would be interesting to evaluate the effect of these proteins on the structure of Es and GEs, and their potential relationship with in vitro lipid digestion.

PUFAs are prone to lipid oxidation. Among other strategies, the addition of water-soluble pro-oxidants or antioxidants has been effective in limiting oxidation processes [10]. Carob fruit extracts contain condensed tannins (T) in the form of non-extractable proanthocyanidin (NEPA), primarily known for either their antioxidant activity [11] or hypolipemic properties [12]. Furthermore, polyphenols (both extractable and NEPA) have health benefits when consumed thanks to their capacity to decrease oxidative stress, and they have been shown to ameliorate some chronic diseases in which insulin resistance is produced [13]. Therefore, the incorporation of carob extract in the systems would have the double function of preventing lipid oxidation and inhibiting pancreatic lipase activity, thus promoting health when consumed.

The objective of the present work was to get a deeper insight into the structure–functionality relationship of n-3 PUFA-enriched Es or GEs as fat substitutes depending on the emulsifying protein (EP), and the addition of T with known antioxidant and hypolipemic properties.

2. Materials and Methods

2.1. Materials and Reagents

Extra virgin olive oil (Carbonell Virgen Extra; SOS Cuétara SA, Madrid, Spain), linseed oil (Natursoy S.L.; Alimentos Ecológicos, Castellterçol, Spain) and fish oil (Omevital 18/12 TG Gold; Cognis GmbH, Illertissen, Germany) were used as lipid phases in emulsion preparations. SC (Excellion EM 6; *Friesland Campina* DMV, Veghel, the Netherlands) and WPI (Provon 295; Glanbia Nutritionals, Kilkenny, Ireland) were used as emulsifying proteins (EPs). Condensed tannins (T) extracted from carob fruit were obtained from Biosearch S.A. (Granada, Spain). Bovine gelatin (200–220 bloom) was obtained from Manuel Riesgo, S.A. (Madrid, Spain), κ-carrageenan from Trades S.A. (Barcelona, Spain) and MTG from Ajinomoto (Tokyo, Japan). All other reagents were of analytical grade and acquired from Panreac Quimica, S.A. (Barcelona, Spain).

2.2. Methods

2.2.1. Formulation and Preparation of Es and GEs

The composition of the emulsions and their process of elaboration were optimized in previous tests to obtain appropriate physicochemical characteristics [14]. Firstly, Es were prepared by mixing four different aqueous phases with SC or WPI (added at optimal emulsification amounts of 2 and 6 g/100 g, respectively) in the presence or not of T (3.9 g/100 g emulsion), and with a lipid phase consisting in a mixture of olive oil, linseed oil and fish oil (44.4, 37.9 and 17.7 g/100 g, respectively), in accordance with Delgado-Pando et al. [7]. Coarse emulsions were elaborated by the dropwise addition of the lipid phase (50 g/100 g) into a homogenizer (Thermomix™ 31, Vorwerk España M.S.L., S.C, Madrid, Spain) containing the different aqueous phases (50 g/100 g) while mixing at 3250 rpm for 20 min at room temperature. The coarse emulsions were passed once through a high-pressure homogenizer (GEA Niro Soavi MODEL Panda Plus 2000, Parma, Italy) at 55/7 MPa (first-stage pressure/second-stage pressure) to obtain fine emulsions. The same procedure was used to prepare the emulsions with T, which were added after each emulsion preparation and subsequently mixed. Es prepared with SC or WPI are referred to as E-SC-C and E-WPI-C, respectively, and used as controls, whereas those containing SC or WPI with added T are referred to as E-SC-T and E-WPI-T.

To structure or gel the different Es, κ-carrageenan (0.3 g/100 g emulsion) and bovine gelatin (0.5 g/100 g emulsion) were dissolved in deionized water (20 mL/100 g emulsion) at 80 °C and then added into 100 g of the emulsions, which were stirred at 1625 rpm/37 °C in the Thermomix, to avoid a possible thermal shock. Then, MTG (1.5 g/100 g emulsion) was dispersed in deionized water (10 mL/100 g emulsion) and added to the mixture. Aliquots of 80 mL were rapidly filled into 100 mL containers and thereafter stored at 4 °C for 24 h to obtain two GEs with SC or WPI (GE-SC-C and GE-WPI-C), both used as controls, and another two containing T (GE-SC-T and GE-WPI-T). Emulsions were stored in refrigeration (4 °C ± 2) until they were used.

2.2.2. Droplet Size Distribution of Es

After diluting between 8- and 10-fold with distilled water, the particle size and distribution of oil droplets in the Es were determined with a Malvern Mastersizer S laser diffraction particle size analyzer (Malvern Instrument Ltd., Worcestershire, UK) equipped with a He-Ne laser (λ = 633 nm). The refractive index of the disperse phase was 1.53 and the refractive index of the dispersion liquid (distilled water) was 1.33. The measurement range was 0.05–900 μm. Obscuration was in the range of 8–15%. Particle size calculations were based on the Mie Scattering theory. Particle size measurements are reported as the volume-weighted mean or volumetric diameter, d_{43} (μm). Measurements were done at least in triplicate.

2.2.3. Rheological Measurements

The rheological properties of both Es and GEs were carried out with a Kinexus pro rotational rheometer (Malvern Instruments Ltd., Worcestershire, UK), equipped with a cone and plate geometry (4° cone angle, 40-mm diameter) for measuring Es, and with a parallel-plate geometry (20-mm diameter and 1-mm gap) for GEs. Before measurement, both Es and GEs were tempered at ambient temperature. GEs were cut from graduated plastic tubes into disk-shaped slices, 20-mm in diameter and 1-mm thick, with a stainless steel cell specially designed for this diameter [15]. All samples were allowed to rest for 15 min before analyses to ensure both thermal and mechanical equilibrium at the time of measurement. Samples were covered with a thin film of Vaseline oil (PRS-Codex, Barcelona, Spain) to avoid evaporation. The temperature was controlled to within 0.1 °C by a Peltier element in the lower plate kept at 25 °C, except when non-isothermal heating processes were carried out. All measurements were done at least in triplicate.

Steady Shear Rheological Measurements of Es

The steady shear measurements were performed for shear rates ranging from 0.1 to 100 s^{-1}. Data from the flow curves were fitted to the power law model ($\sigma = K\dot{\gamma}^{n}$), where σ (Pa) is the shear stress, K (Pa s^{n}) is the consistency index and n is the flow index. Viscosities at 10 and 100 s^{-1} (η_{10} and η_{100}) were also derived from flow curves and compared to K values from power law fits, which correspond to the apparent viscosity at a shear rate of 1 s^{-1} (η_1).

Dynamic Rheological Measurements of GEs

To determine the linear viscoelastic (LVE) region, stress sweep tests were run at 1 Hz with the shear stress (σ) of the input signal varying from 0.02 to 200 Pa. Frequency sweep tests were run to obtain the mechanical spectra of GEs. Samples were subjected to strain that varied harmonically with time at variable frequencies from 0.01 to 10 Hz. The strain amplitude was set at $\gamma = 0.5\%$ within the LVE range. The complex modulus (G^*), storage modulus (G'), loss modulus (G''), and loss factor, tan δ, were determined as functions of frequency. Creep and recovery tests were also conducted on GEs. For this purpose, an instantaneous stress σ_0 (ranging between 1.91 and 35.1 Pa, depending upon formulation), corresponding to 0.5% shear strain within the LVE range, was applied for 600 s in the creep tests and the resulting change in strain over time $\gamma(t)$ was monitored. When the stress was released, recovery was also recorded for 600 s. The creep and recovery results were described in terms of the shear compliance function, $J(t) = \gamma(t)/\sigma_0$. From $J(t)$ data, the relaxation modulus $G(t)$ was obtained and used to find the gel strength (S) and relaxation exponent (n) [16]. Finally, temperature sweep tests were performed for dynamic thermomechanical analysis from 20 to 80 °C at a linear heating rate (2 °C min^{-1}). Frequency (1 Hz) and strain $\gamma = 0.5\%$ (within the LVE range) were fixed.

2.2.4. Simulated In Vitro Gastrointestinal Digestion of Es and GEs

The digestion process in a human gastrointestinal tract was simulated through an in vitro static digestion model, which was a modified version of that described by Laparra et al. [17]. Ten g of Es or chopped GEs were accurately weighed and transferred to Erlenmeyer flasks in triplicate. Then, 90 mL of distilled water were added and the mixture was acidified to pH 2 with 0.1 M HCl. For the gastric step, pepsin (0.2 mg/mL) was added, the volume was adjusted to 100 mL, and the flasks were placed in a thermostatic bath at 37 °C for 2 h with continuous shaking. The gastric step was stopped by placing the flasks in an ice bath and then the pH was adjusted to 6.5 with 1 M $NaHCO_3$. The intestinal phase of digestion was then initiated by the addition of a pancreatin (50 µg/mL)–bile solution (300 µg/mL) and incubation in a thermostatic bath at 37 °C for 2 h with continuous shaking; subsequently, the reaction was stopped by again placing the flasks in an ice bath. The final pH of the solution was adjusted to 7.2 with 0.5 M NaOH. For NEPA from T and antioxidant activity determinations, the remainder-undigested gel was removed by filtering through mesh of 0.16 mm in

diameter, and the filtrate concentrated by freeze-drying. Simulated in vitro gastrointestinal digestion protocols commonly separate the soluble fraction by filtering through Whatman no. 1 filter paper or centrifuging, in order to evaluate accessibility. By filtering the digest through the 0.16 mm mesh, released T are also being considered, and they are expected to play a relevant beneficial role after colonic fermentation [18]. For the analysis of lipid hydrolysis, the digested whole sample was used for lipid extraction, which was performed as described below.

Released Non-Extractable Proanthocyanidins (NEPA) from T and Antioxidant Activity

NEPA extraction was carried out from the freeze-dried digested according to the procedure described by Pérez-Jiménez et al. [19]. Ten mL of butanol/HCl (97.5:2.5, *v*/*v*) containing 0.7 g of $FeCl_3$ were added to this residue and heated at 100 °C for 60 min. Then, samples were cooled to room temperature and centrifuged at 509× *g* for 15 min and the supernatant was collected. The residue was re-extracted with 5 mL of the above solution twice and all extracts were combined to obtain the total amount of 25 mL. The absorbance was measured at 555 and 450 nm in a Shimadzu UV-1800 spectrophotometer (Shimadzu Inc., Kyoto, Japan). Standard curves were obtained for carob pod tannin concentrate. The results were expressed as mg of NEPA released/100 g emulsion.

An automated photochemiluminescent system (Photochem©, Analytik Jena Model AG; Analytic Jena USA, The Woodlands, TX, USA), which measures the capacity to quench superoxide free radicals, was used to determine the antioxidant activity in the NEPA extracts. In brief, 20 µL of the extract containing NEPA were added to reagent kits provided by the manufacturer and the system measured the total antioxidant capacity. Trolox was used as a standard, the samples were measured in triplicate and results were expressed in Trolox Equivalents (mmol TE/g sample).

Extent of Lipolysis during In Vitro Digestion

Once in vitro digestion was finished, fat was extracted following the method described by Cofrades et al. [20]. Briefly, the resulting digest was mixed twice with chloroform/methanol (1:1, *v*/*v*) (Sigma-Aldrich, Madrid, Spain). The organic phase was collected and then purified using a chloroform/methanol/0.9% NaCl solution mix (vol:vol, 3/48/47). Finally, the obtained solution was dehydrated by filtering it through anhydrous sodium sulfate (Merck, Madrid, Spain), evaporated in rotavapor at 40 °C, and subjected to nitrogen stream for 3 min until the isolated lipid phase was obtained. High Performance Size Exclusion Chromatography (HPSEC) was performed to elucidate the triglycerides (TGC), diglycerides (DGC), monoglycerides (MGC) and free fatty acids (FFA) composition and contents, in both the non-digested and digested samples, as described by González-Muñoz et al. [21]. Fifteen mg of extracted fat diluted in 1 mL of tetrahydrofuran were applied in HPSEC (Agilent 1100 series, Madrid, Spain) with a 20 µL sample loop. A refractive index detector (Agilent Technologies 1260 infinity, Madrid, Spain) and two serially-connected 300 mm × 7.5 mm i.d. (5 µm particle size), 0.01 and 0.05 µm, PL gel columns (Agilent, Bellefonte, PA, USA) were operated at 40 °C. HPLC grade tetrahydrofuran was used as the mobile phase with a flow of 1 mL/min. TGC, DGC, MGC and FFA were quantified as g/100 g of sample. The specific refractive index increment (dn/dc value) observed between the sample and the solvent was used for the different compounds' quantitation after accepting equal chromatographic response for all triglyceride-derived compounds. The content of the different groups of compounds, expressed as percentage on oil, were calculated as follows: $100*(A/\sum)$ in which A is the area of the corresponding peak and $\sum$ is the sum of areas for all peaks. In order to verify the peak assignation, standard retention times of TGC, DGC, MGC, and FFA were obtained after injecting an iso-molecular mix of tripalmitin, triestearin, triolein and trilinolein in the case of TGC; dipalmitin, distearin, diolein, dilinolein in the case of DGC; monopalmitin, monostearin, monolein and monolinolein in the case of MGC; and palmitic, stearic, oleic and linoleic acids for FFA. The three samples from each group were measured in duplicate.

2.3. Data Analysis

For rheological and droplet size measurements, two-way analysis of variance (ANOVA) was performed to study the main effects separately: emulsifying protein (EP), presence of condensed tannins (T) and the interaction effect (EP × T). As a significant interaction effect was observed in most of the rheological parameters evaluated, one-way ANOVA was performed, comparing the means of the same T level (without and with) for the two EPs and of the same EP for both presence and absence of T. In addition, for lipid composition, NEPA and antioxidant activity after in vitro digestion, the effect of cold gelling systems (E versus GE) was also studied by using the unpaired Student's *t*-test. Minimum significant differences were calculated using Fisher's least significant difference (LSD) test at 5 and 1% levels. Analyses were done using IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, NY, USA).

3. Results and Discussion

3.1. Droplet Size Distribution of Es

Figure 1 shows the droplet size distribution of E-SC-C and E-SC-T (Figure 1a) and of E-WPI-C and E-WPI-T (Figure 1b). As may be observed in Figure 1, both control Es exhibited a monomodal distribution with a mainly large-sized population (around 1.4 μm), which was narrower and better defined in the case of E-SC-C, likely reflecting a more stable emulsion. Similar results were observed by Jiang et al. [22] in caseinate-based emulsions elaborated with increasing low molecular weight emulsifier concentrations, but exhibiting a bimodal size distribution at a concentration below 0.25% *w/w*. These findings indicate that in control Es, both EPs, SC and WPI, were adequately adsorbed onto the droplets' surface, forming an interfacial membrane through repulsive strong interactions (e.g., steric and electrostatic interactions) between oil droplets, which are resistant to rupture [8,23].

On the other hand, the incorporation of T produced larger droplets with an increase in the width of distribution, thus significantly changing the profile of the droplet size distribution in the case of both EPs. More specifically, E-SC-T (Figure 1a) showed a bimodal pattern with two main similar populations of droplets with sizes of around 1 and 45 μm, respectively. Both distribution peaks could be related to individual oil droplets and aggregates of oil droplets, respectively. In turn, E-WPI-T exhibited one larger-sized population around 1.4 μm, as also observed for E-WPI-C (Figure 1b), and two minority populations with peaks at around 20 and above 100 μm, respectively. Hence, the presence of T might interfere between the oil droplets and the protein as a filler, therefore decreasing the interfacial tension and producing in some way self-aggregation of protein molecules, causing coalescence and/or flocculation with larger-size droplets. According to Trujillo-Cayado et al., [24] the occurrence of a second population of oil droplets could be the consequence of recoalescence phenomena during the high-pressure homogenization process.

Particle size measurements d_{43} were also obtained for all the four Es. The d_{43} values were significantly lower for E-SC-C (1.23 ± 0.084 μm) than for E-WPI-C (1.70 ± 0.285 μm). The faster the emulsifier adsorption, the smaller the droplets of the emulsion formed [22]. The d_{43} value increased very significantly with additional T incorporation in Es prepared with both EPs (23.8 ± 0.829 and 16.6 ± 2.87 μm, respectively). However, the contrary was observed in the control Es, where d_{43} was significantly higher in the E-SC-T sample than in the E-WPI-T one. A previous study showed that phenolic compounds mainly interact with casein rather than with whey protein [25], which is reflected by a much higher aggregate size of casein globules implying strong hydrophobic bindings of T with caseinates. In Es stabilized by SC and WPI, Chang and McClements [6] reported lower and similar $d_{4,3}$ values between them (0.283 ± 0.003 and 0.225 ± 0.006 μm, respectively) than those found in this study, although differences could be ascribed to the different formulation and conditions of preparation of the Es made by these authors.

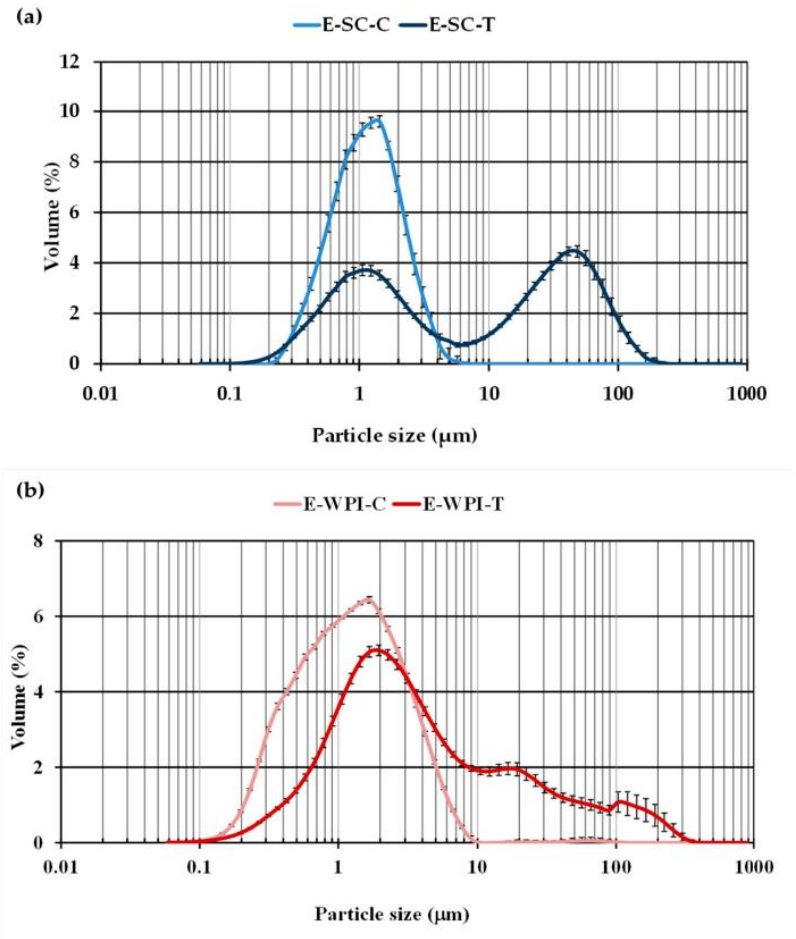

Figure 1. Droplet size distributions for liquid-like emulsions (Es): (**a**) stabilized by SC without and with added condensed tannins (E-SC-C and E-SC-T); (**b**) stabilized by WPI without and with added condensed tannins (E-WPI-C and E-WPI-T).

As a result, SC and WPI seem to be good emulsifiers, and therefore they could form stable and homogeneous emulsions, although it is likely that they show a different rate of lipid digestibility [6]. Furthermore, the presence of T yielded higher mean diameters and different droplet size distributions, indicating that T incorporation could lead to the occurrence of coagulation or coalescence phenomena, mainly in the case of Es prepared with SC, due to the higher affinity of polyphenols for this milk protein. In addition, these structural changes produced by T presence in the formulations could have consequences on expected antioxidant and hypolipemic activities.

3.2. Rheological Properties of Es and GEs

3.2.1. Steady Shear Rheological Properties of Es

The flow behavior of an emulsion is critical to its physical stability [22]. Figure 2 illustrates the applicability of the power law model to the Es stabilized by both SC and WPI, either without or with added T. Shear stress–shear rate plots become almost linear when plotted on double logarithmic coordinates, and the power law model properly described the data of Es, which exhibited a shear-thinning behavior. However, the emulsion E-WPI-T became more viscous, showing the highest resistance to flow and the highest shear stress value over the complete shear rate range studied, and even with an apparent

and measurable initial yield stress at~3 Pa, indicating plastic behavior. E-WPI-T also had apparent viscosity values similar to those reported by Jiang et al. [22] in caseinate-based Es stabilized with low molecular weight emulsifiers. On the contrary, small differences can be observed between the flow curves of E-SC-C, E-SC-T and E-WPI-C samples, mainly at the highest shear rates.

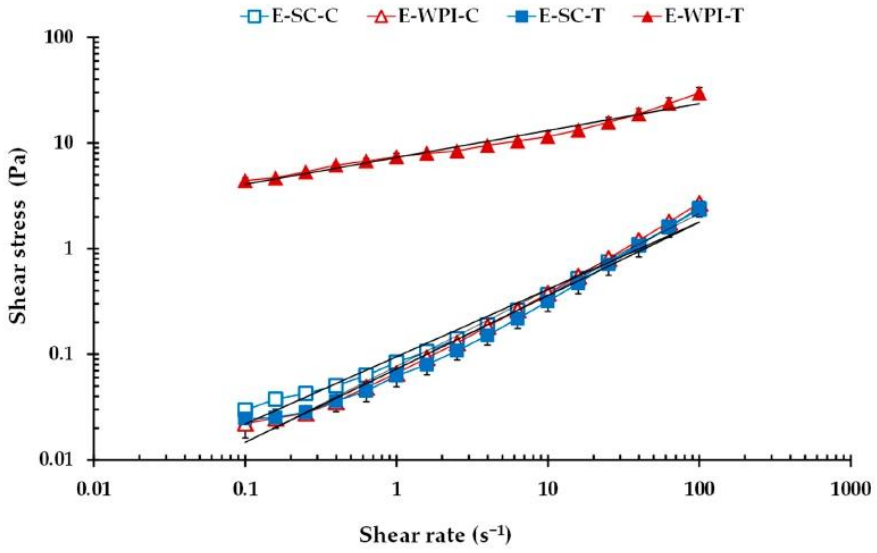

Figure 2. Typical flow curves and applicability of the power law model to liquid-like emulsions (Es) stabilized by two emulsifying proteins (SC and WPI), without (E-SC-C and E-WPI-C) and with added condensed tannins (T) (E-SC-T and E-WPI-T).

The parameters K and n are determined from the plots of log σ versus log $\dot{\gamma}$, where each resulting straight line's intercept is log K and the slope is n. Table 1 shows all the steady shear parameter values of Es. Based on the R^2 determination coefficient values, E-WPI-T exhibited the worst power law fit, probably due to the fact that the power law model disregards yield stress. With regard to the effect of the EP used in the formulation, in both Es without and with added T, there were significant differences ($p < 0.05$) between the K, n, η_{10} and η_{100} values of the two emulsions, although the effect was much more significant in emulsions with added T. Therefore, structural differences of Es formulated with different EP are greatly magnified when the bioactive compound is added, which shows the importance of the type of EP in the final characteristics of the system, which may ultimately condition a greater or lower effect of the bioactive compound.

Table 1. Effect of emulsifying protein (EP) and condensed tannins (T) on the steady shear rheological properties of liquid-like emulsions (Es).

Liquid-like Emulsions (Es)	K (Pas^n)	n (-)	R^2	η_{10} (Pa s)	η_{100} (Pa s)
E-SC-C	0.095 ± 0.001 ^{Aa}	0.637 ± 0.010 ^{Bb}	0.984 ± 0.002	0.036 ± 0.001 ^{Ba}	0.024 ± 0.001 ^{Ba}
E-WPI-C	0.077 ± 0.001 ^{Bb}	0.723 ± 0.001 ^{Aa}	0.988 ± 0.004	0.038 ± 0.001 ^{Ab}	0.027 ± 0.000 ^{Ab}
E-SC-T	0.073 ± 0.015 ^{Ba}	0.696 ± 0.017 ^{Aa}	0.979 ± 0.005	0.031 ± 0.004 ^{Ba}	0.024 ± 0.003 ^{Ba}
E-WPI-T	7.22 ± 0.272 ^{Aa}	0.250 ± 0.019 ^{Bb}	0.957 ± 0.009	0.849 ± 0.0.073 ^{Aa}	0.296 ± 0.039 ^{Aa}

Values are given as mean ($n = 3$) ± standard deviation. A, B: effect of EP. For each rheological property and the same T level (without or with), mean values without the same letter are significantly different ($p < 0.05$). a,b: effect of T incorporation. For each rheological property and the same EP, mean values without the same letter are significantly different ($p < 0.05$). K and n: consistency and flow behavior indexes from the power law model, respectively; η_{10} and η_{100}: viscosities at 10 and 100 s^{-1} from flow behavior curves; (-): dimensionless.

E-SC-C showed significantly higher K and lower n values than E-WPI-C, although it also showed significantly lower η_{10} and η_{100} values. However, E-WPI-T sample presented the highest K, η_{10} and η_{100} values, and E-SC-T the lowest ones. In addition, all the Es showed pseudoplastic behavior ($n < 1$), but mainly E-WPI-T brought about a significant lowering of the flow index (n), that is to say, the values were further removed from Newtonian behavior ($n = 1$), indicating higher pseudoplasticity and greater resistance to the flow. In accordance with Pal [26], an increase in droplet size is accompanied by a decrease in the degree of shear thinning behavior in concentrated emulsions, as observed in this study for E-SC-T as compared to E-WPI-T (Table 1). In contrast, other authors observed that with increasing cluster size, the viscosity increases due to an increase in effective oil volume fraction [27].

According to the T effect, in samples prepared with WPI, the incorporation of T significantly increased the values of K, η_{10} and η_{100}, and significantly decreased the n value (Table 1). On the contrary, the incorporation of T in samples prepared with SC only had a significant effect on the n value, and both E-SC-C and E-SC-T had weaker, looser structures than their WPI counterparts. Therefore, in Es, the effect of the presence of T was dependent on the EP used to stabilize the emulsion. The much lower viscosity of E-SC-T compared to E-WPI-T could be attributed to the fact that with SC and T, there were fewer interactive emulsion droplets due to increased particle size.

A liquid-like protein-stabilized emulsion is characterized by its oil volume content, its droplet size distribution and the protein surface coverage, which forms an immobile viscoelastic adsorbed layer, at the oil–water interface [4]. Significant differences in the behavior of E-SC-C and E-WPI-C emulsions must be related to the protein system used to stabilize the Es, as well as to droplet size distribution, because the lipid material was the same in all cases. Both SC and WPI emulsifiers are soluble/dispersible mixed milk protein ingredients, although SC is a mixture of flexible proteins, whereas WPI is a mixture of globular ones [6].

On the other hand, the T used in our experiments contain 34–48% NEPA and 0.5–1% soluble extractable polyphenols [12], and a high affinity between polyphenolic compounds of crude extracts and casein has been previously observed [25]. Therefore, it is likely that the T that interacted with the SC protein remained in the protein matrix. On the contrary, the authors just cited observed very little interaction between WPI and pure phenolic compounds. Additional functional ingredients may also lead to long-term transient gelation by the depletion flocculation mechanism [28], which could justify the existence of a yield stress in the E-WPI-T sample, and in its resultant weak gel-like structure. A solid-like emulsion gel may be generated from a stable liquid-like emulsion by gelling the continuous phase and/or aggregating the emulsion droplets, and aggregated droplets may be present if there is an excess of unadsorbed protein (depletion flocculation) [29].

3.2.2. Dynamic Rheological Properties of GEs

Frequency Sweep Tests

It is useful to determine dynamic rheological properties of GEs to study the contribution of the EP to emulsion stability and the effect of droplet–droplet interactions on the viscoelasticity of the system [30]. Mechanical spectra of GE-SC-C, GE-WPI-C, GE-SC-T and GE-WPI-T are shown in Figure 3. All the GEs exhibited solid-like elastic-dominant behavior because storage modulus (G') was higher than loss modulus (G'') over the entire frequency range. This result indicates that the cold gelling system used, a mixture of gelatin, κ-carrageenan and MTG, produced the formation of interactive and viscoelastic gel systems in all the Es. It is well known that MTG forms GEs with enhanced rheological properties and stability by covalent cross-linking by acyl transfer between glutamine and lysine residues in proteins [3,14,31]. Therefore, GEs can be classified as weak physical gels with predominantly solid-like character [32].

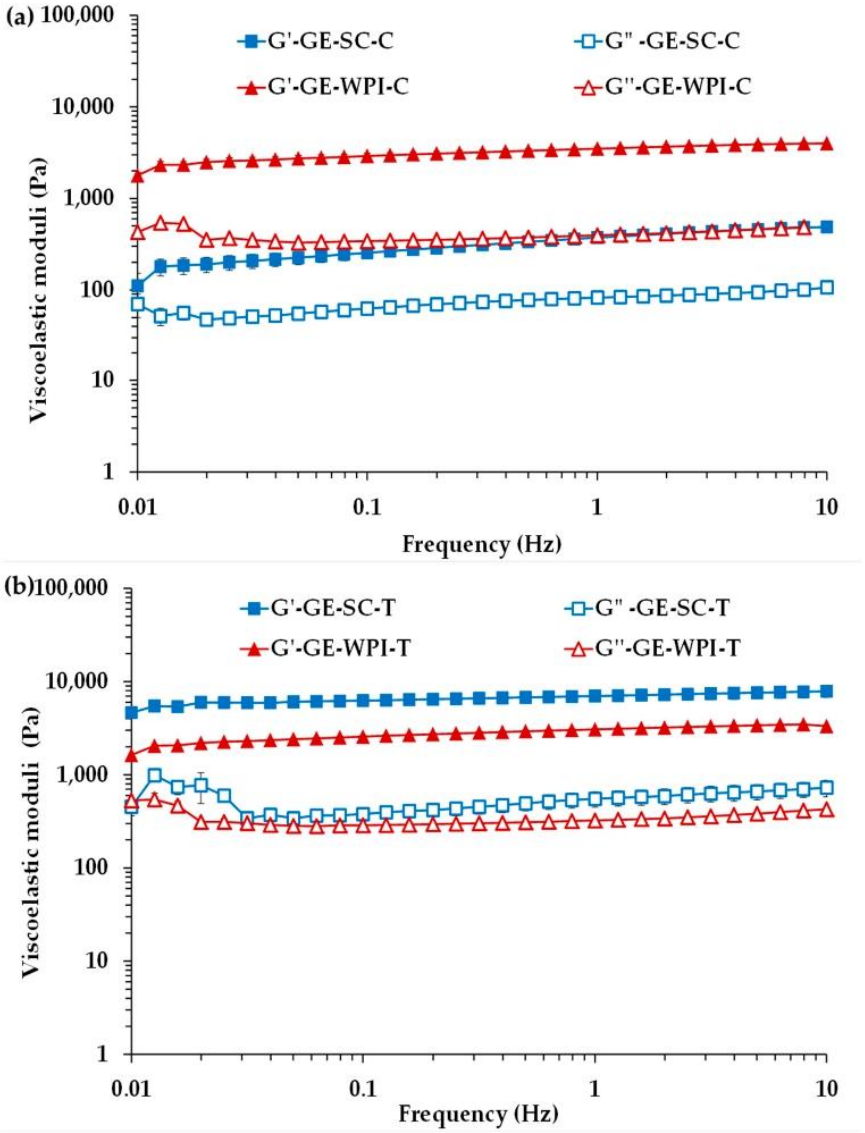

Figure 3. *G′* and *G″* as functions of frequency for gelled emulsions (GEs): (**a**) stabilized by SC and WPI without added condensed tannins (GE-SC-C and E-WPI-C); (**b**) stabilized by SC and WPI with added condensed tannins (GE-SC-T and GE-WPI-T).

Moreover, in all GEs, *G′* was practically frequency-independent, whereas *G″* exhibited a tendency to increase at lower frequencies (from 0.1 to 0.01 Hz). This increase was even more remarkable in the presence of T (Figure 3b). The relative *G″* increases observed between 0.1 and 0.01 Hz could be attributed, at least partially, to a possible denaturation of the proteins in the structured systems because of longer durations of the shear. Then, they may unfold their secondary and tertiary structure, allowing the absorption of water molecules, so they could swell, and an increase in the *G″* values, therefore causing the three-dimensional network to lose its stability. This can be explained by a partial disruption of the adsorbed protein layer that stabilized the network, which was more significant in the GEs with added T. A greater accessibility of glutamine and lysine residues by MTG due to structure unfolding could also contribute to the observed increase in *G″*.

The effects of EP and T incorporation on dynamic rheological properties at 1 Hz are shown in Table 2. GE-WPI-C showed significantly higher G' and G'' values and lower tan δ ones than GE-SC-C. This last result is in accordance with the fact that the gap between G' and G'' was greater in the GE-WPI-C sample than in the GE-SC-T one (Figure 3a). Therefore, GE-WPI-C, having a higher arginine content than its SC counterpart [14], exhibited a more strongly connected structure and higher viscoelasticity. Molecular weights of monomeric forms of casein proteins (15–26 kDa) are quite similar to those of the major proteins from WPI (18–66 kDa) [33,34]. However, when compared with whey proteins, caseins are particularly disordered and considerably hydrophobic, which facilitates their rapid adsorption during emulsification and leads to a rapid formation of a thick sterically stabilizing layer that protects against flocculation and coalescence [34], resulting in much lower viscoelasticity.

Table 2. Effect of emulsifying protein (EP) and condensed tannins (T) on dynamic rheological properties (at 1 Hz), weak gel model parameters and creep rheological properties of gelled emulsions (GEs).

Gelled Emulsions	G' (Pa)	G'' (Pa)	tan δ (-)	A (Pa $s^{1/z}$)	z (-)	R^2	S (kPas^n)	n	R^2
GE-SC-C	373 ± 36.5 ^{Bb}	82.4 ± 6.93 ^{Bb}	0.221 ± 0.003 ^{Aa}	375 ± 38.3 ^{Bb}	7.09 ± 0.669 ^{Bb}	0.990 ± 0.009	0.514 ± 0.122 ^{Bb}	0.222 ± 0.022 ^{Aa}	0.993 ± 0.004
GE-WPI-C	2485 ± 53.0 ^{Ab}	278 ± 4.15 ^{Ab}	0.114 ± 0.001 ^{Ba}	2508 ± 54.5 ^{Ab}	13.1 ± 0.603 ^{Aa}	0.998 ± 0.001	1.79 ± 0.372 ^{Ab}	0.185 ± 0.034 ^{Aa}	0.984 ± 0.018
GE-SC-T	6990 ± 937 ^{Aa}	552 ± 102 ^{Aa}	0.079 ± 0.004 ^{Bb}	7025 ± 102 ^{Aa}	19.8 ± 0.231 ^{Aa}	1.000 ± 0.000	5.57 ± 1.85 ^{Aa}	0.083 ± 0.013 ^{Bb}	0.982 ± 0.014
GE-WPI-T	3813 ± 209 ^{Ba}	411 ± 23.4 ^{Aa}	0.109 ± 0.001 ^{Aa}	3840 ± 209 ^{Ba}	14.1 ± 0.233 ^{Ba}	0.999 ± 0.000	5.01 ± 0.507 ^{Aa}	0.141 ± 0.034 ^{Aa}	0.996 ± 0.002

Values are given as mean ($n = 3$) ± standard deviation. A, B: effect of EP. For each rheological property and the same T level (without or with), mean values without the same letter are significantly different ($p < 0.05$). a, b: effect of T incorporation. For each rheological property and the same EP, mean values without the same letter are significantly different ($p < 0.05$). G', storage modulus; G'', loss modulus; tan δ, loss factor (= G''/G'). A, "interaction strength"; z, "coordination number"; S, gel strength; n, relaxation exponent; R^2, determination coefficient; (-): dimensionless.

On the other hand, when T were incorporated in the emulsions, both structured GEs had viscoelastic moduli values that were much higher than in their respective control counterparts (Table 2). In addition, this increase was much more significant when SC was used as emulsifier. Therefore, it seems that T addition caused a structural reinforcement, being especially notable in GE-SC-T, as it was also evidenced by its significantly lower tan δ value reflecting higher viscoelasticity and more elastic gel-like behavior. Note that tan δ values, which indicate the amount of energy lost to the amount of energy stored, were lower than 1 but close to 0.1; this result also reflects a weak gel behavior. On the other hand, interactions between T and SC were stronger than between T and WPI, as reported previously [14,25]. In similar cold-set GEs, these authors reported that casein proteins, rich in proline and other apolar amino acids and with a more open random structure, are more prone to interact with T than with WPI.

In addition, GEs can also be characterized using the concept of weak gel [35]. According to this weak gel model, weakly structured food systems can be characterized by a three-dimensional network where rheological units are bound by weak interactions [36]. In this study, rheological units would represent cross-links between milk protein molecules, and between T and proteins in GEs with added T, assuming that the contribution to the rheological properties of the cold gelling system was the same in all systems. The dynamic data for these systems are described by a power law Equation (1) relating dynamic complex modulus (G^*) and frequency (f):

$$G^*(f) = Af^{1/z} \quad (1)$$

where z is the "coordination number", which is the number of flow units interacting with one another within the three-dimensional network, and it can be assumed as an extent of interactions [36], and A is

a constant that can be interpreted as the "interaction strength". It is worth noting that the A parameter is equal to the complex modulus (G^*) evaluated at a frequency (f) of 1 Hz.

Values of A and z obtained from fits of G^* values to power laws between 10 and 0.1 Hz are also shown in Table 2. Data between 0.1 and 0.01 Hz were discarded because the observed G'' increase in this frequency decade would significantly affect the linearity of G^* versus frequency. Strength and development of GEs networks were influenced by both the EP and T effects studied. As it could be expected from the elastic gel-like behavior observed, the A and G' values of all the GEs were very similar, and with the same significant differences between them (Table 2). Therefore, the strength of the interactions was also significantly weaker ($p < 0.05$) in control GEs than in those with added T. This result reflects that T increase the effectiveness of protein adsorption, decreasing protein content in the continuous phase, and making the network more resistant to permanent deformation. In addition, the effect was mainly significant in GE-SC-T, also evidenced by a greater extent of interactions (higher z value) reflecting a higher stability of GE-SC-T, as indicated above. This reinforcement caused by the interaction between T and SC could increase the thickness of the protein viscoelastic film around the fat droplets, influencing lipid digestibility as described below. There were no significant differences between the z values of WPI GEs with and without added T, meaning a similar number of interactions and degree of organization (Table 2). Nevertheless, the significantly lower developed strength of interactions of GE-WPI-T as compared to GE-SC-T could imply that in the system GE-WPI-T, T were located far from the lipid phase [14].

Han et al. [25] also reported that casein is more prone to interact with polyphenols than whey proteins. On the other hand, conformational changes such as structural unfolding of caseins could allow greater reactivity of MTG with glutamine and lysine [33] increasing viscoelasticity and improving the conformational stability of this gelled emulsion.

Creep and Recovery Tests

Creep-recovery analysis is a transient test performed at constant stress within the LVE range. It produces creep and recovery compliances $J(t)$ over longer-time scales than oscillatory rheological measurements [37], and consequently these experiments can cause irreversible breakdown of short-range interactions. Hence, they can provide additional information of the contribution of the type of EP and the addition of T on $J(t)$ and, therefore, on the network stability over longer-time scales than oscillatory tests.

Figure 4a,b show the mean creep-recovery compliances $J(t)$ for GEs without and with added T, respectively. GE-SC-C (Figure 4a) showed much higher values of compliance $J(t)$ than GE-WPI-C during both the creep and recovery stages. This indicates that during loading, there was more sequential rupturing of cross-links (probably weak interactions) in GE-SC-C than in the GE-WPI-C gelled system. In addition, the GE-WPI-C sample showed a more complete recovery (Figure 4a), indicating that it was a more elastic network, as it is also evidenced by its lower tan δ values (Table 2).

The addition of T (Figure 4b) changed this rheological behavior pattern completely. First, both GEs showed much lower $J(t)$ values than those of their control counterparts, corroborating the structural reinforcement produced by T incorporation, especially in GE-SC-T showing lower $J(t)$ values than GE-WPI-T. These data corroborate those observed from the frequency sweeps. However, note that samples with added T also showed less recovery upon load removal (Figure 4b). This fact would indicate that for longer loading times, GEs with added T could suffer more structural collapse and irreversible breakage of interactions, which is associated with poorer binding properties [14].

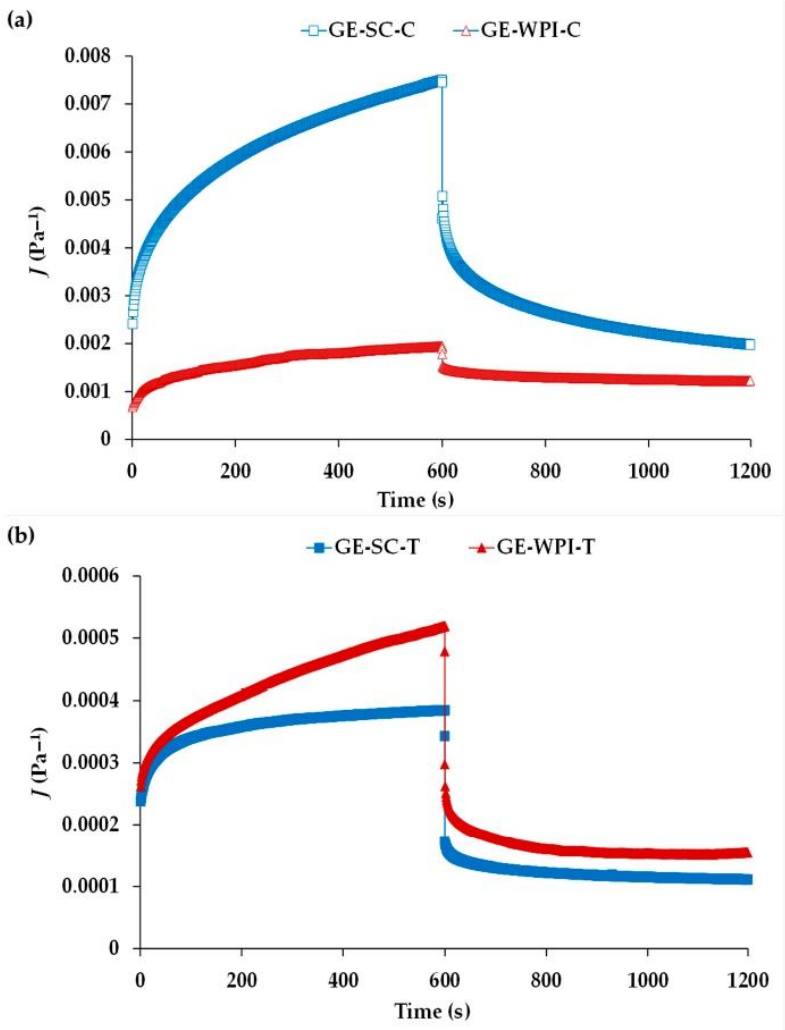

Figure 4. Evolution of creep and recovery compliance $J(t)$ values for gelled emulsions (GEs): (**a**) stabilized by SC and WPI without added condensed tannins (GE-SC-C and E-WPI-C); (**b**) stabilized by SC and WPI with added condensed tannins (GE-SC-T and GE-WPI-T).

Data from Figure 4 make it possible to derive the relaxation modulus $G(t)$ [37], which provides the "gel strength" (S) and "relaxation exponent" (n) by means of Equation (2):

$$G(t) = S.t^{-n} \quad (2)$$

As indicated previously, T produce stronger GEs, as also shown by their significantly higher S values as compared to their control counterparts (Table 2), making these gelled systems more resistant to deformation, especially in the case of using SC as EP. This suggests that both EPs (SC and WPI) formed an adsorbed layer around the oil droplets, likely of different thickness, because the proteins unfold and rearrange their different secondary and tertiary structures to expose hydrophobic residues to the hydrophobic phase [23], which allows interaction with its neighboring protein molecules to form this adsorbed layer. Hence, the stability of the GEs depends on the different structure of the adsorbed protein. T could modify the structure of the adsorbed protein or act as a filler between this layer and the droplets, because of the affinity that polyphenols show towards food proteins [25].

This is very remarkable in the case of SC, so that the presence of T completely changes the structural configuration from a flexible and weak network to the most rigid and stable protein network, as shown by their higher *S* value and lower *n* one (Table 2). The lower the *n* value, the higher the density of physical cross-linking reactions, which increases the extension of the junction zones and the degree of connectivity, also indicating a better organized and cohesive network [38]. Therefore, T improved the structural stability in the presence of SC more significantly, which may be explained by a stronger interaction of this protein with polyphenols than that of WPI [25]. The results confirm the effects of T on the strength of interactions between rheological units.

In summary, the above results reflect the stronger and more elastic network with a high degree of connectivity of GE-SC-T ensuring that the nature of interactions in the protein network is preserved.

Temperature Sweep Tests

Figure 5 shows the influence of temperature within the LVE range in terms of storage (*G′*) and loss moduli (*G″*) to analyze the temperature stability of GEs. As these GEs are intended to be used as functional fat replacers in different food matrices suffering thermal treatment during their processing, their thermo-rheological behavior is crucial in terms of their applicability. The thermal profile of GE-SC-C (Figure 5a) indicated a gel weakening with increasing temperature, as reflected by a decrease in both *G′* and *G″* from 20 up to 80 °C. Nevertheless, GE-WPI-C (Figure 5b) showed a much more stable profile, keeping *G′* one order of magnitude greater than *G″* over the whole temperature range.

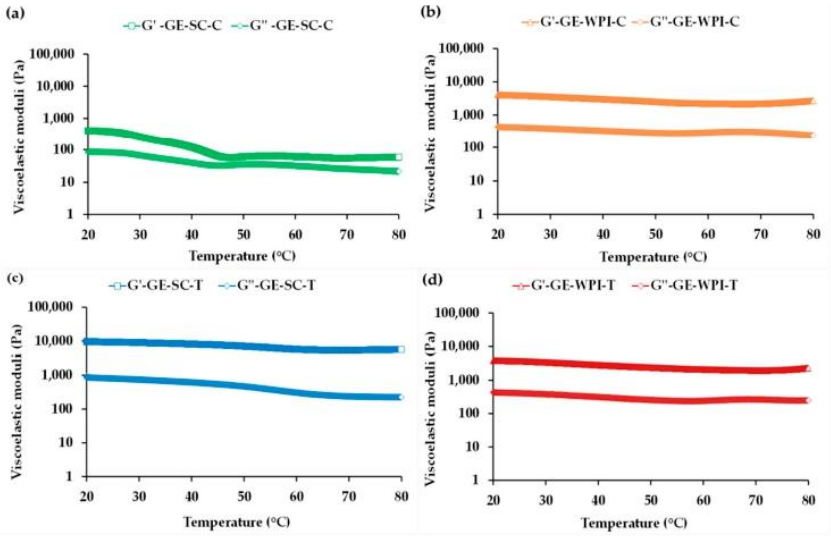

Figure 5. Temperature sweeps of *G′* and *G″* for gelled emulsions (GEs): (**a**) stabilized by SC without added condensed tannins (GE-SC-C); (**b**) stabilized by WPI without added condensed tannins (GE-WPI-C); (**c**) stabilized by SC with added condensed tannins (GE-SC-T); (**d**) stabilized by WPI with added condensed tannins (GE-WPI-T).

In turn, in the case of GEs with added T, GE-SC-T (Figure 5c) showed a more pronounced decrease in *G″* than GE-WPI-T (Figure 5d), but in both structured systems temperature hardly affected gel elasticity. It is worth noting that the WPI profiles are very similar in the absence and presence of T.

This result evidences that, with the exception of GE-SC-C, in all other GEs there is only a slight loss of structural configuration by the rupture of weak intermolecular bonds between the adsorbed proteins as temperature increases. Therefore, mainly GE-SC-T, but also GE-WPI-C and GE-WPI-T, may be suitable as fat replacers in foods, including meat products, subject to high temperatures.

3.3. Simulated In Vitro Gastrointestinal Digestion of Es and GEs

3.3.1. Released Non-Extractable Proanthocyanidins (NEPA) from T and Antioxidant Activity

The amount of NEPA released (accessible to be fermented by microbiota and accordingly absorbed) at the end of in vitro gastrointestinal digestion, as well as the corresponding antioxidant activity, are shown in Table 3. As expected, the four systems without added T (E-SC-C, E-WPI-C, GE-SC-C and GE-WPI-C) showed no NEPA content. However, they did present some antioxidant activity, which is mainly attributed to proteins and peptides released during digestion processes, although the contribution of antioxidants from the oils cannot be discarded. Samples with added T (E-SC-T, E-WPI-T, GE-SC-T and GE-WPI-T) showed a significantly higher amount of NEPA and antioxidant activity ($p < 0.05$) than their respective controls, and thus it can be deduced that NEPA was the main cause of the antioxidant activity of either Es or GEs, although T interactions with other components could affect the accessibility of this ingredient, and therefore its antioxidant properties [11].

Table 3. Non-extractable proanthocyanidins (NEPA) released (mg/100 g) and antioxidant activity (mg Equation Trolox/100 g) after simulated gastrointestinal in vitro digestion of the different emulsions developed.

Added T		E-SC	E-WPI	GE-SC	GE-WPI
mg NEPA released/100 g E or GE	C	-	-	-	-
	T	432 ± 44^{Ax}	344 ± 18^{Bx}	235 ± 12^{Ay}	167 ± 8^{By}
mg Equation Trolox released/100 g E or GE	C	152 ± 11^{Axb}	148 ± 39^{Axb}	104 ± 6^{Byb}	140 ± 5^{Axb}
	T	1396 ± 162^{Axa}	1060 ± 110^{Bxa}	580 ± 28^{Aya}	485 ± 36^{Bya}

Values are given as mean ($n = 3$) ± standard deviation. Different letters in the same row (A, B) indicate significant differences ($p < 0.05$) as a function of EP (SC, WPI) for each emulsion structuration level (E, GE). Different letters in the same row (x, y) indicate significant differences ($p < 0.05$) as a function of cold gelation (E, GE) for each EP (SC, WPI). Different letters in the same column (a, b) indicate significant differences ($p < 0.05$) as a function of T (C, T) for each system studied.

With regard to the effect of emulsion structuration by cold gelation, the digests from Es exhibited a significantly ($p < 0.05$) higher NEPA content and much higher antioxidant activity than their gelled counterparts GEs. Therefore, NEPA were more effectively retained within the gelled matrices, which impaired their release. This is largely attributed to an incomplete release of T from GEs during digestion, as some debris was observed in these samples when the digests were filtered.

Discussing the effect of EP on control systems, only GE-WPI-C had significantly ($p < 0.05$) higher antioxidant activity than its GE-SC-C counterpart. On the contrary, regarding T-systems, the two systems elaborated with SC (E-SC-T and GE-SC-T) had a significantly higher amount of NEPA and antioxidant activity than their counterparts formulated with WPI (Table 3), suggesting a different behaviour of samples when T were incorporated to the system, which is in line with the rheological results. Therefore, as can be seen in the table, 100 g of E or GE will provide ≈ 450–250 or ≈ 350–150 mg NEPA, respectively, depending on the EP (SC or WPI), which would reach the colon in free form, exerting their antioxidant capacities and being able to be fermented by microbiota. It is well known that NEPA exert an important biological action in the colon, improving antioxidant and antiproliferative capacities, reducing intestinal tumorigenesis and modifying gene expression, as has been observed in different animal models [19]. Moreover, NEPA are extensively fermented by the action of microbiota, giving place to absorbable metabolites, which are also involved in interesting systemic effects [18].

Finally, in all the systems, the presence of T improved ($p < 0.05$) their antioxidant activities very significantly (Table 3), as was expected, corroborating previous findings [11]. Therefore, T incorporation is a convenient strategy for enhancing antioxidant capacity and retarding lipid oxidation in these systems.

3.3.2. Extent of Lipolysis in Es and GEs

Table 4 summarizes lipid composition after in vitro digestion of the four liquid-like emulsions (Es), and the four gelled emulsions (GEs), with and without T. The extent of lipolysis was affected by the EP used, by the formulated system, and finally by T incorporation. Hence, the three factors must be taken into account.

Table 4. Lipid composition after in vitro digestion of liquid-like and gelled emulsions (Es and GEs). as a function of emulsifying protein (EP), condensed tannins (T) incorporation and cold gelation.

Lipid Composition	Added T	E-SC	E-WPI	GE-SC	GE-WPI
TGC	C	63.6 ± 1.32	64.4 ± 2.56 ^{xb}	63.0 ± 1.22 ^{Aa}	48.0 ± 5.90 ^{Byb}
	T	64.1 ± 1.59 ^{Bx}	68.5 ± 1.96 ^{Axa}	59.6 ± 1.57 ^{yb}	55.4 ± 2.37 ^{ya}
DGC	C	13.4 ± 0.50 ^{Axa}	16.1 ± 1.41 ^{Ba}	10.2 ± 0.91 ^{Ay}	17.8 ± 1.40 ^{Ba}
	T	10.7 ± 0.04 ^{Bb}	13.6 ± 1.57 ^{Ab}	11.0 ± 0.59 ^{A}	12.2 ± 0.69 ^{Bb}
MGC	C	4.77 ± 0.52 ^{Ayb}	3.74 ± 0.20 ^{By}	5.72 ± 0.13 ^{Axb}	8.58 ± 1.54 ^{Bx}
	T	6.28 ± 0.74 ^{Aa}	3.39 ± 0.31 ^{By}	6.73 ± 0.32 ^{Aa}	8.26 ± 0.49 ^{Bx}
FFA	C	18.3 ± 1.08 ^{Ay}	15.8 ± 1.28 ^{By}	21.1 ± 0.29 ^{Ax}	25.7 ± 3.54 ^{Bx}
	T	18.9 ± 0.89 ^{A}	14.5 ± 1.40 ^{By}	22.7 ± 2.53	24.2 ± 1.68 ^{x}

Values are given as mean (n = 3) ± standard deviation. Different letters in the same row (A, B) indicate significant differences ($p < 0.05$) as a function of EP (SC, WPI) for each emulsion structuration or cold gelation level (E, GE). Different letters in the same row (x, y) indicate significant differences ($p < 0.05$) as a function of cold gelation level (E, GE) for each EP (SC, WPI). Different letters in the same column (a, b) indicate significant differences ($p < 0.05$) as a function of T (C, T) for each system studied. TGC: triglycerides; DGC: diglycerides; MGC: monoglycerides; FFA: free fatty acids.

The effects of the delivery system (Es or GE) and EP (SC or WPI) are discussed together since they are interconnected. The comparison of lipid profile from formulated systems (Es and GEs) revealed higher fat digestion in GEs, especially in the case of those containing WPI, showing less triglycerides (TGC) or more free fatty acids (FFA) than their Es counterparts, in both C and T samples. In a previous paper [20], we demonstrated that gelatin and MTG do not have any impact on pancreatic lipase activity; hence, the promotion on lipolysis by GEs should be attributed to their structural characteristics. Regarding the effect of EP, SC and WPI showed significant differences in lipolysis but with opposite trends depending on the delivery system used. Thus, Es containing SC (with and without T) showed a greater extent of lipolysis than those with WPI, as can be observed in the significantly lower levels of diglycerides (DGC) observed compared with E-WPI-C (−16.8%, $p < 0.01$), along with higher levels of monoglycerides (MGC) and FFA (21.6% and 13.6%, respectively; $p < 0.01$). Such differences would be partially explained because of the smaller droplet size (lower $d_{4,3}$) found in E-SC as compared with E-WPI, which would have allowed a better access of pancreatic lipase to the emulsified lipid in the surface of SC-coated droplets than in that of WPI-coated droplets. Similarly, the initial SC layer surrounding the droplets did not prevent the formation of FFA in corn oil-in-water emulsions [39]. In addition, the results obtained by Borreani et al. [40] also showed that lipase was able to access the emulsified lipid more readily in a conventional emulsion with calcium caseinate-coated droplets than in other cellulose ether-coated droplets. Similar results were found in emulsions made with SC as emulsifier, which showed a greater lipid digestion than the whole oil [20]. On the contrary, regarding GEs, GE-SC-C was more resistant to digestion, with a significantly higher TGC level (23.9%, $p < 0.001$) and lower DGC, MGC and FFA levels (74.6%, 50% and 21.9%, respectively) than GE-WPI-C. A suitable explanation of this result is that offered by Pang et al. [41], who showed that gelatin can significantly modify GE properties as a function of pH and temperature, showing a greater loss of structure in those emulsions made with WPI. Taking these data into account, it is possible that the higher lipid digestion found in GE-WPI-C is due to a greater system rupture in the in vitro gastric stage. Furthermore, it can be observed that when gelatin is not used (E-WPI-C), fat digestion is lower

compared with GE-WPI-C and also compared with E-SC-C, confirming the pH gelatin-weakness hypothesis. The described rheological properties differ with lipid digestion results; as it has been pointed out, GE-WPI-C samples showed lower values of compliance and a more complete recovery, with a high MTG-gel-strengthening effect, along with more elastic behavior compared to GE-SC-C (Figure 4a). However, following the conclusions of Pang et al. [41], the cause can be related to gastric rheological changes due to pH-sensitivity, which has not been studied this time. Furthermore, it must be remembered that the in vitro digestion protocol chosen could be considered as an initial phase of the intestinal stage. Thus, a gastric rupture of GE-WPI-C would promote a faster fat digestion at this early point than GE-SC-C would.

Apart from the type of EP and the formulated systems, T addition is another important factor to consider. The results obtained clearly indicate a different effect of T on lipolysis depending on the emulsifying protein (EP) used. In this sense, in the systems containing SC, T addition involved the promotion of fat digestion, and did not display the expected pancreatic lipase inhibition [12,13]. As can be seen in Table 4, E-SC-T showed a significantly lower amount of DGC and a higher amount of MGC compared with the E-SC-C sample, while GE-SC-T presented significantly lower TGC and higher MGC levels than GE-SC-C. The lack of the expected hypolipemic effect of T, when incorporated to SC-T systems, could be connected with T-SC interactions. The necessary interaction of T and lipase to cause its inhibition may be sterically hindered if T remain bound to SC. On the other hand, the higher fat digestion found in SC-T systems compared to SC-control ones, which should be linked to the important structural changes caused by T addition, as the rheological results demonstrated, remains to be explained. As shown above, the incorporation of T to E-SC decreased viscosity with respect to E-SC-C, facilitating enzyme digestion access. Regarding GE-SC-T results, with the greatest lipolysis among SC-systems, they can be due to the rheological behavior pattern. In connection with the previous discussion (Figure 4b), GE-SC-T showed less recovery, suggesting more risk of structural collapse and irreversible breakage of interactions, which can be responsible for the observed lipid profile. Finally, digestion results concur with NEPA released in SC-T systems, as they show the highest amount of NEPA in free form, indicating an easier digestion.

In contrast to SC, both systems elaborated with WPI and T (E-WPI-T and GE-WPI-T) displayed lipid digestion reduction, as compared to those without T. This delay in lipid digestion may be the result of two causes: (a) structural changes due to the addition of T; and (b) a direct T inhibitory effect on pancreatic lipase. Both E-WPI-T and GE-WPI-T systems exhibited a significantly higher amount of TGC, and lower amount of DGC, as compared with their counterparts without added T (Table 4), although the effect of T was more pronounced in the GE system. The results suggest different behavior patterns regarding structural changes. Firstly, E-WPI-T presented pseudoplastic properties with higher viscosity and resistance than E-WPI-C. For its part, the GE-WPI-T sample presented high deformability (Figure 4b), evidencing a greater rupture of cross-links that could facilitate droplets to become flocculated or coalesced in the gastrointestinal tract. Then, the surface area of lipids exposed to proteolytic enzymes is reduced, thus impairing fat release and slowing down lipid digestion. Concerning lipase inhibition, it is important to point out that NEPA release in WPI-samples was lower than in SC-samples (Table 3). However, in WPI-samples, when NEPA were released, it appears that they were capable of interacting with and blocking lipase. This would not happen in SC samples due to the interaction between SC-T, as detailed above. In this sense, the lack of the expected hypolipemic effect of T, when incorporated into SC-T systems, could be connected to T-SC interactions. The necessary interaction of T and lipase to cause its inhibition may be sterically hindered if T remain bound to SC.

Lastly, T-systems have been compared among each other. E-SC-T showed significantly higher lipid digestion than E-WPI-T, while both GE-T systems (GE-SC-T and GE-WPI-T) displayed a similar degree of lipid digestion, counterbalancing the strong difference between their control counterparts.

4. Conclusions

The results obtained make it possible to conclude that the emulsifying protein and/or addition of the bioactive compound (T) significantly modify the structure of the systems, the release of the bioactive compound, the antioxidant activity and lipid digestion. Formulating the system using SC as the emulsifying protein cancels the inhibiting effect of T on pancreatic lipase activity completely. Due to their structural/rheological characteristics, SC-T systems present greater lipid digestion compared with the controls, which implies an opposite effect from that expected. Thus, it is of great importance to verify that bioactive compounds maintain their potential effect once added to a system or particular matrix in order to formulate a functional food. The election of the system to be used will be adapted to the objective pursued. Accordingly, if the purpose is to develop a system able to release a healthy type of fat (e.g., rich in n-3 PUFAs), the system to be chosen would be GE-WPI-C, since it allows greater lipid digestion. If T were added in search of an antioxidant effect, it should be considered that fat would be digested to a lesser extent due to the lower bioavailability of the GE-WPI-T system. On the other hand, E-SC-T would be the most adequate option if the goal is to diminish the absorption of a certain type of fat (e.g., rich in SFAs) and to increase the antioxidant activity in the digestive tube of the consumer.

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Capítulo 2

Actualización de los conocimientos sobre el consumo de carne y productos cárnicos sobre diferentes aspectos de la salud, con especial énfasis en los mecanismos de oxidación. Papel de los cárnicos funcionales

Antecedentes: La bibliografía disponible hasta la fecha señala una relación entre el consumo de carne roja y procesada con un mayor riesgo de padecer algunas enfermedades crónicas muy prevalentes, principalmente ECV, cáncer y DMT2 (Aune y cols., 2009; Richi y cols., 2015). Sin embargo, en muchos de los trabajos descritos no se cita la cantidad o el tipo de carne consumido, refiriéndose en la mayoría de los casos a cifras muy elevadas (Celada y cols., 2016; Celada & Sánchez-Muniz, 2016). Además, la carne y los productos cárnicos son una excelente matriz para incluir compuestos bioactivos, lo que permitiría formular cárnicos funcionales capaces de minimizar el posible impacto perjudicial de su consumo, al mismo tiempo que mejorar sus propiedades nutricionales (Jiménez-Colmenero y cols., 2001; Olmedilla-Alonso y cols., 2013).

Objetivos:

- Definir el concepto de carne y productos cárnicos según las principales agencias reguladoras, así como su contribución a la dieta actual.
- Describir el impacto de un elevado consumo de carne y productos cárnicos, con especial hincapié en los productos de oxidación originados y su posible implicación sobre la salud.
- Analizar el concepto de cárnico funcional. Revisar y discutir la asociación establecida entre un elevado consumo de carne y productos cárnicos y el estrés oxidativo.
- Evaluar la formulación de cárnicos funcionales con potencial efecto antioxidante en el procesado, almacenamiento y cocinado. Valorar las propiedades funcionales de dichos cárnicos sobre el estatus antioxidante de los consumidores (tanto en modelos animales y ensayos clínicos).
- Discutir los efectos del consumo de PACs sobre la microbiota, permeabilidad intestinal e inflamación. Evaluar el impacto de su consumo sobre algunas de las patologías crónicas más prevalentes.

Publicación 4

Can Meat and Meat-Products Induce Oxidative Stress?

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Antioxidants

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Resumen: De acuerdo al Glosario de términos del *Codex Alimentarius*, la carne se define como la porción comestible de cualquier mamífero. Esta amplia definición nos da una idea de la complejidad y la problemática asociada al consumo de carne. Por ello, en este trabajo se revisa el concepto de carne y productos cárnicos por las principales agencias reguladoras internacionales, así como algunos aspectos de la clasificación de la carne blanca/roja, insistiendo en la necesidad de crear una clasificación más precisa y evitar las generalizaciones.

De forma general, se acepta que la carne es un alimento rico en AGS y colesterol, siendo estos componentes los responsables de algunas de las opiniones negativas que reciben. Una elevada ingesta de carnes, especialmente de carnes rojas, se asocia positivamente con el desarrollo de enfermedades cardiovasculares y algunos tipos de cáncer. El factor común que podría ayudar a comprender esta relación es el papel del estrés oxidativo en la etiología y/o progresión de estas enfermedades, ya que la naturaleza altamente perecedera de la carne los convierte en una fuente importante fuente de productos de oxidación durante su producción, almacenamiento, cocinado y consumo (digestión y metabolización). Los principales sustratos susceptibles a la oxidación son los lípidos, especialmente los AGP de cadena larga, los cuales originan malondialdehído, 4-hidroxi-nonenal y oxisteroles. Además, también se puede formar compuestos de oxidación de origen proteico como los carbonilos proteicos; por la combinación de un azúcar reductor y un grupo amino, formando productos finales de glicosilación avanzada; o finalmente, también se puede originar óxido de trimetilamina, comúnmente conocido como TMAO, tras la metabolización de algunos aminoácidos por la microbiota. La ingesta elevada de todos estos compuestos, puede inducir estrés oxidativo per se o incrementar la formación de especies reactivas de oxígeno, contribuyendo notablemente a la progresión de enfermedades crónico-degenerativas.

En el presente artículo resumimos cómo se forman los compuestos de oxidación en la carne y productos cárnicos, así como la implicación de su consumo sobre la salud. Además, destacamos la necesidad de realizar estudios de intervención controlados que prueben diferentes tipos y cantidades de carne y productos cárnicos para determinar con precisión la relación entre su consumo, la oxidación y las enfermedades degenerativas. Con ello, se pretende minimizar el impacto negativo que está recibiendo la carne y resaltar la importancia de una dieta plural y equilibrada.

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antioxidants

MDPI

Review

Can Meat and Meat-Products Induce Oxidative Stress?

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Abstract: High meat and meat-products consumption has been related to degenerative diseases. In addition to their saturated fatty acids and cholesterol contents, oxidation products generated during their production, storage, digestion, and metabolization have been largely implicated. This review begins by summarizing the concept of meat and meat-products by the main international regulatory agencies while highlighting the nutritional importance of their consumption. The review also dials in the controversy of white/red meat classification and insists in the need of more accurate classification based on adequate scores. Since one of the negative arguments that meat receives comes from the association of its consumption with the increase in oxidative stress, main oxidation compounds (malondialdehyde, thermaloxidized compounds, 4-hydroxy-nonenal, oxysterols, or protein carbonyls) generated during its production, storage, and metabolization, are included as a central aspect of the work. The review includes future remarks addressed to study the effects meat consumption in the frame of diet–gene interactions, stressing the importance of knowing the genetic variables that make individuals more susceptible to a possible oxidative stress imbalance or antioxidant protection. The importance of consumed meat/meat-products in the frame of a personalized nutrition reach in plant-food is finally highlighted considering the importance of iron and plant biophenols on the microbiota abundance and plurality, which in turn affect several aspects of our physiology and metabolism.

Keywords: meat; metabolism; oxidation; oxidative stress

1. Introduction

Currently, food, in addition to being a source of energy, nutrients and bioactive compounds assuring growth and development, is perceived as a key factor that influences body functions and may help to prevent some degenerative diseases [1]. Although diet composition and quality significantly differ between countries and/or World regions, meat has generally been considered a central food item

that once consumed, assures several nutritional compounds needed for the correct homeostasis at any age. Nonetheless, despite being an excellent source of nutrients and bioactive compounds, there are epidemiological concerns linking excessive meat consumption with increased risk of various chronic diseases, such as cardiovascular diseases (CVD) and some types of cancer, particularly colorectal cancer [2,3]. However, recent research data highlight the need to performed accurate meta-analysis collecting and pooling dietary data from different cohorts analyzing each diet-disease pair after adjusting for same confounders [4].

In addition, the relationship between meat consumption and degenerative diseases has been wedged by making inadequate generalizations, forgetting that "meat" is an ample concept that refers to the edible part of any mammal, as we have to take into account that mammal is also an ample concept covering terrestrial and marine mammals. The last one includes animals such as seals and whales, which normally are not consumed Western countries. In fact, it has to keep in mind that cross-sectional studies, performed at a certain time, cannot be directly used to relate cause and effect. More than 50 years ago, Hill [5] listed nine types of major evidences that could help reaffirm the results of observational studies: consistency, specificity, temporality, biological gradient, plausibility, coherence, experimentation and analogy. The Hill's postulates are accepted and considered by both those who are in favor as for those who are in against using observational studies to draw conclusions about causation. Thus, the possible negative effects of meat are highly dependent on the nutritional status of consumers and the plurality of the dietary compounds eaten together with meat [2,6]. For instance, many experiments designed to mimic the nutrient loads in current westernized diets did not include potential biologically active protective compounds present in whole foods, limiting the evidence to confirm a mechanistic link between the intake of red meat as part of a healthy dietary pattern and colorectal cancer risk [7]. Although different mechanisms have currently been suggested to explain this association, the impact of meat consumption, especially red meat, is still a debate within the scientific community [7]. However, the common factor that could help to understand this relationship is the role of oxidative stress in the etiology and/or progression of these diseases [8].

Meat is a food rich in protein, lipids, and heme iron, whose composition makes it highly perishable as a consequence of the chemical and enzymatic processes that take place during production and storage. Lipids and proteins are susceptible to oxidation, being responsible for the loss of organoleptic and nutritional properties in this food type [9]. Additionally, the presence of transition metals such as heme iron greatly contributes to the formation of these undesired products. Among the oxidized products of lipid origin are malondialdehyde (MDA), 4-hydroxy-nonenal (4-HNE), 4-hydroxy-hexenal (4-HHE) or oxysterols, while those originating from proteins are mainly protein carbonyls. However, fat triglycerides in meat in presence of metal transition and oxygen suffer oxidation, oligomerization and hydrolysis opening discussion in this important topic [10]. The gastrointestinal tract is the gateway for oxidized food compounds produced during processing and storage of meat, but they can also originate during digestion and metabolization. Specifically, the stomach appears to be an excellent environment for meat lipid peroxidation, having observed a greater formation of lipid hydroperoxides, MDA and 4-HHE both in the stomach content and in plasma or urine after meat consumption [11–13]. Some studies have shown that the amount of oxidation compounds reaching the colon is approximately 50% less than that available at the small intestine, which is clearly indicative of a significant absorption rate [8]. The available literature indicates that a part of the diet oxidation compounds is absorbed in the lymph or directly into the bloodstream, being able to induce oxidative damage in biological systems [14,15].

Furthermore, in the frame of the precision diet/nutrition concept, a strong effort is being made to find out how the most convenient food items should be combined to assure good functionality and to decrease the degenerative disease risk of any given person considering the genoma-diet interaction [16–18]. Given this great interest, very active research is being performed to find out about, develop, and test numerous functional foods aimed at increasing the nutritional value and exerting some beneficial effects on certain diseases [19–21]. For this, it is first necessary to know the oxidative

processes that can take place in meat and meat-products from their processing to its consumption. The present manuscript has the objective to describe the main oxidative processes that take place in meat and meat-products during their production, storage, cooking, digestion, and metabolization, which may be involved in the origin and development of more prevalent chronic degenerative diseases.

2. Meat and Meat-Products

Before starting to deal with the subject of this in-depth review, it is necessary to know the definition of meat concept by the main regulatory agencies at the European and American levels. According to the Codex Alimentarius Glossary [22], meat is the edible part of any mammal. This general definition gives a clear idea of the complexity of the topic as various parts of different various mammals differing in age, familiae, gender, species, varieties, feeding, etc., can be included in this general definition. For this reason, we would like to introduce a more detailed definition by the European Commission and the American Code of Federal Regulations.

Due to the extensive terminology on meat and meat-products, the American Meat Science Association (AMSA) developed in 2018 a standardized lexicon of terms that allows the precise description and classification of meat and meat-products to minimize confusion when transmitting meat-related information. Thus, AMSA defines meat as "skeletal muscle and its associated tissues derived from mammalian, avian, reptilian, amphibian, and aquatic species harvested for human consumption. Edible offal consisting of organs and non-skeletal muscle tissues also are considered meat" [23]. However, several authorities have their own definitions about meat and meat products, most times addresses to define meat and/or meat products normally consumed in each country's sphere of influence.

Thus, the European Commission, according to Regulation (EC) No 853/2004 [24], understands by meat "edible parts of the animals referred in these three groups (*sic*): (a) Fresh meat of domestic and wild ungulates; (b) Game meat, and (c) Meat-products".

(a) Fresh meat of domestic and wild ungulates: refers to meat that has not undergone any preserving process other than chilling, freezing or quick-freezing, including meat that is vacuum-wrapped or wrapped in a controlled atmosphere. The definitions of "meat" and "fresh meat" are laid down in Annex I of Regulation (EC) No 853/2004. Fresh meat of domestic and wild ungulates includes fresh meat of for example: bovine animals (including buffalo and bison); ovine and caprine animals; porcine animals; solipeds.

(b) Game meat: game animals refer to land mammals and birds—either in the wild or farmed—that are not normally considered to be domestic animals (the following animals are specifically excluded: bovines, domestic swine, sheep and goats, domestic solipeds, domestic fowl, turkeys, guinea-fowl, ducks, and geese). Fresh game meat must fulfil the animal health requirements laid down in the legislation applicable to each classification of game animal. This classification is based on both the species of animal and its origin. Therefore, there is a clear distinction made between: fresh meat from wild game, and fresh meat from farmed game.

(c) Meat-products: are defined as processed products, resulting from the processing of meat or from the further processing of such processed products, so that the cut surface shows that the product no longer has the characteristics of fresh meat.

Instead, the American Code of Federal Regulations does not make these distinctions and defines meat as "the part of the muscle of any cattle, sheep, swine, or goats which is skeletal or which is found in the tongue, diaphragm, heart, or esophagus, with or without the accompanying and overlying fat, and the portions of bone (in bone-in product such as T-bone or porterhouse steak), skin, sinew, nerve, and blood vessels which normally accompany the muscle tissue and that are not separated from it in the process of dressing. As applied to products of equines, this term has a comparable meaning" [25]. This document also defines meat-products, as "any article capable of use as human food which is made wholly or in part from any meat or other portion of the carcass of any cattle, sheep, swine, or goats, except those exempted from the definition as a meat food product by the Administrator in specific

cases or by the regulations in part 317 of this subchapter". Thus, the European Commission and the American Code of Federal Regulations do not include in their meat definitions any reference to marine mammals.

3. Production, Consumption, and Nutritional Importance of Meat and Meat-Products

Meat, meat-products, and other goodness of animal origin have greatly increased in number in the last century, especially during the last six decades all over the World [26]. If we look at the 2017 figures, overall world meat production increased by 1.25% to 323 million tons, with a predominant increase in bovine and poultry meats compared to pork and sheep meat. In fact, annual meat production is projected to increase by about 150 million tons in three decades (from 218 million tons in 1997–1999 to 367 million tons by 2027–2030) [26]. This increase is primarily due to two main factors, the increasing pressure on the livestock sector to meet the growing demand for high-value animal protein and improving productivity in developing countries. According to the Food and Agriculture Organization of the United Nations (FAO) and its database (FAOSTAT) the world's livestock sector has been growing at an unprecedented rate due to a combination of population growth, rising incomes, and urbanization. However, this upward trend is expected to start to slow down, mainly as a result of lower production and consumption in Europe [26]. All these predictions could be clearly modified by health crises such as the current Covid-19.

Taking into account the consumption data of meat and meat-products represented in Table 1, we realize the importance of not generalizing in the concept of meat. Each country presents an eating pattern that is conditioned by reasons of culture and availability, which will directly have an impact on health. Nonetheless, it has to be pointed out that the declared consumption is just apparent and in some way misleading, as the "actual" meat consumption (the meat that we really eat) as that of other dietary compounds (e.g., vegetables) represents 33–50% of the apparent one that consider some not suitable part for consumption or does not take into account just wasting, that tend to be higher in the high income [27].

Table 1. Meat consumption worldwide and by continent in 2017.

Type of Meat	Meat Consumption (kg/per capita/year) *					
	World	Africa	America	Asia	Europe	Oceania
Bovine	9	5.63	27.83	4.68	14	31.22
Mutton and goat	1.86	2.49	0.62	1.93	1.75	10.79
Pork	15.7	1.48	18.65	15.18	35.75	24.24
Poultry	15.18	6.21	41.94	9.71	24.59	43.96
Others	0.84	1.43	0.65	0.55	1.84	2.1
Total	42.58	17.24	89.69	32.05	77.93	112.31

* The per head supply of each such food item available for human consumption is then obtained by dividing the respective quantity by the related data on the population actually partaking of it (FAOSTAT). Most recent data available in this database [28].

The main components of meat are water (60–80%), protein (16–25%) (approximately 40% of its amino acids are essential), and fat (1–30%). There are also small amounts of non-protein nitrogenous substances (free amino acids, peptides, creatine, nucleotides, etc.), carbohydrates, lactic acid, vitamins (thiamine, niacin, retinol, and vitamins B_6 and B_{12}), small amount of vitamin D and minerals (e.g., heme iron and Zn of high bioavailability) and others which are no less important such as P, Se, Na, K y Co [29]. Red meat is also a source of lipoic acid. However, these proportions vary depending on the animal, age, sex, diet, and anatomical areas analyzed [30].

The World Health Organization (WHO) already mentioned that meat and meat-products (sic) "not only provide high-value protein but are also important sources of a wide range of essential micronutrients, in particular minerals such as iron and zinc, and vitamins such as vitamin A". For the large majority of people in the world, particularly in developing countries, livestock products remain a

desired food for nutritional value and taste, being one of the causes of the increase in its production and consumption [31]. It should be noted that meat and meat-products are the main source of vitamin B_6 and the second source of niacin [3]. Thus, the absence of meat in the diet of some countries makes it difficult to achieve the recommended intakes of vitamin B group. However, these figures could not be extrapolated to other world regions since diet and the type of meat consumed varies markedly between different countries (Table 2). Anyhow, its consumption can contribute partially to achieving the recommended dietary intakes of vitamins and minerals [3,32–35].

At present, there exists great concern about the intake of red and/or processed meat, as many studies have associate the intake of these products with the incidence and prevalence of chronic diseases such as obesity, type 2 diabetes mellitus (T2DM), CVD, and different types of cancer [2,3]. As a result of these studies, many health-related agencies have recommended restricting the intake of these products [36]. Nonetheless, we believe that these recommendations, made under the protection of health objectives, are not objective enough and deserve some degree of criticism and discussion, as different publications include [2,37–39]. In fact, some publications demand to perform new meta-analysis collecting and pooling dietary data from different cohorts analyzing each diet-disease pair after adjusting for same confounders [38]; other based in five de novo systematic reviews that considered certainty in the evidence, the magnitude of potential benefits and harms, and explicit consideration of people's values and preferences have recently been made a set of recommendations on red meat and processed meat consumption. The panel recommendations were developed by using the Nutritional Recommendations (NutriRECS) guideline development process and suggest that adults continue current unprocessed red meat consumption (weak recommendation, low-certainty evidence). Similarly, the panel suggests adults continue current processed meat consumption (weak recommendation, low-certainty evidence) [4]. In addition, many epidemiological approaches based on meta-analyses of prospective observational studies adjust for total energy intakes their statistical models do not permitting to define risk in terms of absolute levels of exposure. Thus, the relative risk of change in each component of diet depends on the other components for which it is substituted, fact that is not generally specify in publications. Additionally, the effect size of the individual dietary factors might be overestimated, as the intake of healthy dietary factors are generally positively correlated with each other and inversely correlated with harmful dietary factors. These facts highlight the need to analyze each diet–disease pair, quantifying the effect size after adjusting for same confounders. In addition, most cohorts have been made without considering that suboptimal diet is an important preventable risk factor for non-communicable diseases [38].

Table 2. Nutritional composition of main types of red meat (beef and pork) in USA, UK, and Spain.

	Beef			Pork		
	USA	**UK**	**Spain**	**USA**	**UK**	**Spain**
Energy (kcal)	126	129	131	144	124	155
Protein (g)	21.0	22.5	20.7	21.2	21.8	20.0
Fat (g)	4.0	4.3	5.4	5.9	4.0	8.3
SFA (g)	1.4	1.7	2.2	2.0	1.4	3.2
MUFA (g)	1.6	1.9	2.5	2.7	1.5	3.6
PUFA (g)	0.2	0.2	0.2	0.6	0.7	0.6
Niacin (mg)	6.2	9.7	8.1	4.8	6.9	8.7
Tiamin (mg)	0.1	0.1	0.1	1.0	1.0	0.9
Vitamin B_{12} (μg)	1.5	2.0	2.0	0.7	1.0	3.0
Iron (mg)	1.8	2.7	2.7	0.9	0.7	1.5
Zinc (mg)	3.9	4.1	3.8	2.0	2.1	2.5
Selenium (mg)	26.0	7.0	3.0	32.4	13.0	14.0
Sodium (mg)	54.0	63.0	61.0	54.0	63.0	76.0
Potasium (mg)	323.0	350.0	350.0	384.0	380.0	370.0

Data related to 100 g edible meat. SFA, MUFA, PUFA, saturated, monounsaturated, and polyunsaturated fatty acids. Modified from Delgado-Pando [40].

Oxidation has been defined as an important factor for several chronic degenerative diseases [41]. Due to relatively high iron content of meat and the central role of iron as prooxidant [42], several works have associated red meat consumption with CVD development and some types of cancers, [43–45]. Nonetheless, this relationship has recently been criticized due to unacceptable generalization and because several points still pending verification. Thus, the problem of this association partially derives from the misuse of the terms "red" or "white" meats, being a misleading source due to their generic use both in the general population and in the scientific community. Currently, we still do not know how to differentiate which product could be considered red meat and which could not, and even within the same animal species we can find meat fractions classified as red and others as white. It may seem that the simplest classification in color terms seems also related to the iron content (Table 3). Nonetheless, it has to be taken into account that the amount of heme iron seems associated to the age of animals, as older animals have less moisture, leading any generalization to wrong conclusions. For example, in Spain, although Suckling, Recental, and Paschal lambs are available, due to organoleptic properties such as taste and flavor, the Suckling lamb is by far the most consumed and preferred by the Spanish people, clearly determining differences observed on heme iron between Spain and American ground lamb (Table 3). Furthermore, Spanish lamb heme iron content is rather close to that of Chicken breast clearly accepted as white meat. Moreover, when talking about poultry meat such as turkey, clear location has to be made as myoglobin content is higher in the legs than in the wings, as they hardly fly and their muscular activity is located in the legs, whose greater supply of oxygen is responsible for the redder meat. Thus, the extrapolation of results of heme iron should be carefully done, after considering the age and the meat part of the animals consumed or studied.

Table 3. The label of red and white meat according to iron content.

	Food and Nutrient Database for Dietary Studies				Base de Datos Española de Composición de Alimentos			
	Iron (mg)	Total Fat (g)	SFA (g)	PUFA (g)	Iron (mg)	Total Fat (g)	SFA (g)	PUFA (g)
Iberian ham	14.29	25.0	7.14	1.31	4.3	19.2	7.81	1.18
Pâté	9.19	13.1	4.0	2.46	5.5	29.5	10.48	3.59
Ground beef	1.97	19.07	7.29	0.51	1.9	21	8.51	1.25
Ground lamb	1.78	19.49	8.05	1.39	1.12 *	14.55	5.34	0.74
Ground Pork	1.28	20.6	7.66	1.85	1.3	23	7.43	3.51
Pork sausage	1.2	27.25	8.83	5.12	1.44	28.1	10.55	2.60
Cooked ham	1.0	17.46	6.42	1.67	1.2	5.1	1.9	0.6
Turkey leg	0.83	3.38	1.0	0.91	1.5	8.36	2.6	2.3
Chicken nuggets	0.83	20.36	3.57	6.51	1.39	15.12	2.88	2.3
Chicken breast	0.51	7.67	1.96	0.95	1.5	1.2	0.33	0.28

Red meat and meat-products; White meat and meat-products. * mostly suckling lamb; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; Source: Food and Nutrient Database for Dietary Studies (FNDDS) and Base de Datos Española de Composición de Alimentos (BEDCA). Compositions are expressed either per 100 g edible portion of different meat.

In addition, it has to be pointed out that there are no unique and unified criteria distinguishing red and white meats, since they have been classified according to their myoglobin concentration (heme iron) but also to lipid profile, mitochondrial densities, physiology of muscle fiber and/or in response to physiological changes during metabolism and/or postmortem proteolysis. Therefore, from here we are critical and request to accurately describe the parameter of real interest when communicating scientific information and not using the broad concept of red or white meat [23,46].

However, in relation to oxidation, what is the most determining factor? Current evidence indicates that iron is an important factor in the formation of oxidized compounds through the Fenton reaction, as summarized by Van Hecke et al. [8]. However, if we put on a scale the heme iron implication or the food lipid composition, it would be the availability of oxidizable substrates, such as lipids, that would clearly determine the meat oxidation levels [47–49]. Thus, we insist on the urgent need to perform ample controlled studies considering meat major factors (e.g., level of PUFA, heme iron and different

meat protein with antioxidant/prooxidant properties) to find out a sort of ponderate oxidation score that allows us to classified meat accurately.

4. Meat Consumption as a Source of Oxidative Stress

Oxidative stress has been defined as a critical factor engaged in the origin of most degenerative diseases [41]. Diet has been found to contribute to the pro-oxidant and antioxidant balance as both the deficiency or the high consumption of some foods and nutrients are highly implicated [50].

Although meat cannot be considered a highly oxidized matrix, due to its relatively low PUFA content, meat and meat-products undergo oxidative changes during storage, processing, digestion, and metabolization, which make them a potential source of oxidizing agents. These changes take place from the moment of the animal's slaughter, where the conversion of the muscle into meat already begins to form oxidation compounds. The main substrates susceptible to oxidation are lipids, especially those long-chain PUFAs, which provide the food with variations in its texture, color, flavor, and odor. All these oxidation reactions follow a common process: an initiation stage, in which free radicals are generated; a propagation phase in which the number of oxidized and oxidizing compounds multiplies; and finally, a third stage that is known as a termination, in which the radicals react with each other or with other non-radical compounds (antioxidants) to give rise to non- or less-oxidizing products. The mechanisms involved in lipid oxidation have been elegantly reported elsewhere [51].

On the other hand, a high intake of meat and meat-products can promote reactive oxygen species (ROS) formation at the gastrointestinal tract [14]. Many of these products are generated from lipid hydroperoxide decomposition such as reactive aldehydes, ketones and epoxides, which are cytotoxic [52]. Once formed in the stomach, they are easily absorbed by the intestine to subsequently interact with proteins and lipids to form advanced lipid oxidation end-products [52]. It has been shown that animals and humans, after ingesting peroxidized foods, absorb and excrete large amounts of MDA, 4-HNE, and other carbonyls (Figure 1). The pathological effects of reactive aldehydes are related to their ability to modify reactive proteins or DNA by cross-linking, protein oligomerization, immune responses, and to bind to the receptor for advanced glycation end-products (AGEs), activating the NADPH oxidase and generating ROS [52].

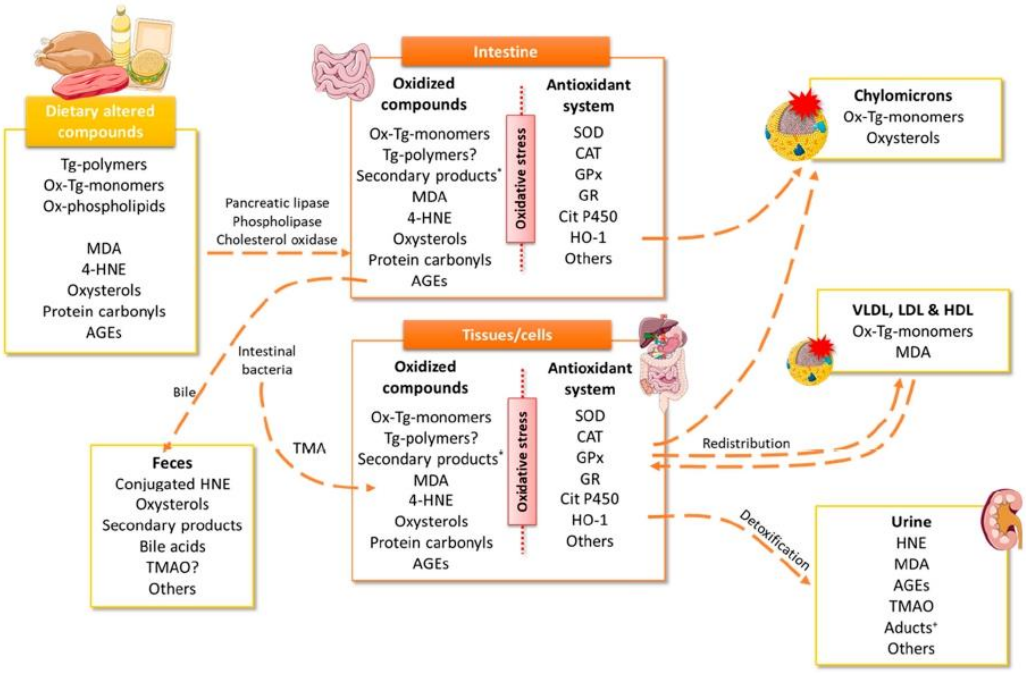

Figure 1. Possible metabolic fate of dietary altered compounds. The scheme illustrates the different body pathways for eliminating those altered products observed. *secondary products included short- or medium-chain alkyl group, carbonyl, hydroxyl, aldehyde, ester, epoxy, carboxyl, etc. $^{+}$Aducts include HNE adducts and HNE conjugates. 4-HNE, 4-hydroxy-nonenal; AGEs, advanced glycation end-products; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; HDL, high-density lipoproteins; HNE, hydroxynonenal; HO, heme oxygenase; LDL, low-density lipoproteins; MDA, malondialdehyde; Ox, oxidized; SOD, superoxide dismutase; Tg, triglycerides; TMAO, trimethylamine N-Oxide; VLDL, very low density lipoproteins.

In this section, the main oxidation compounds generated during the storage, cooking or digestion process will be briefly summarized; among which are MDA, 4-HNE, oxysterols, protein carbonyls, advanced glycation end-products (AGEs), and trimethylamine N-oxide (TMAO), that, once consumed, can induce oxidative stress (Figures 1 and 2).

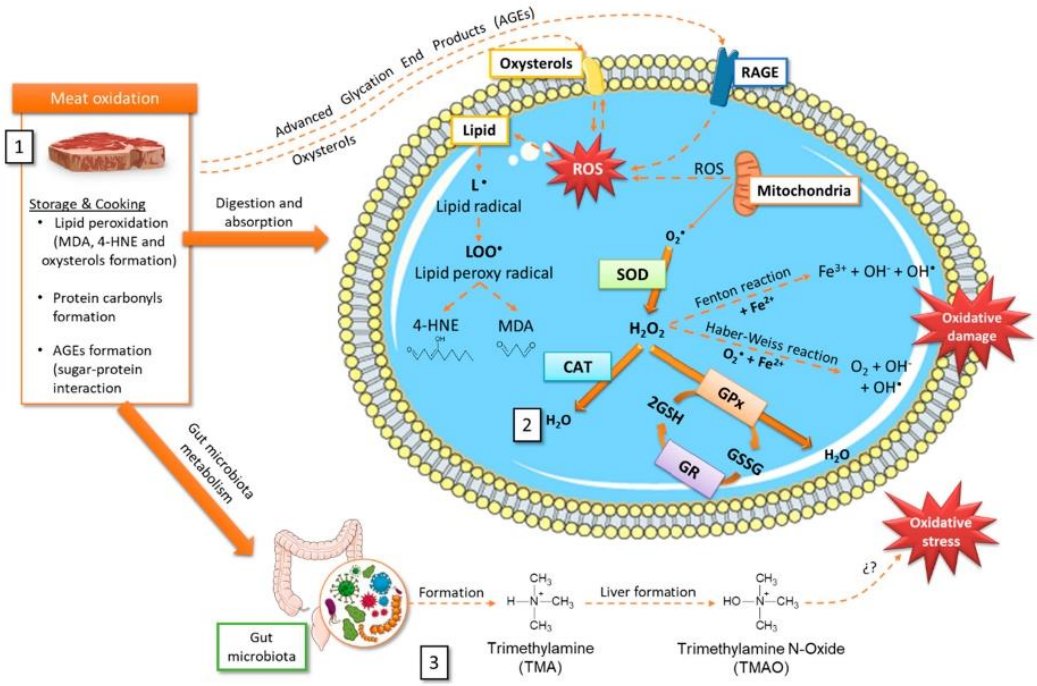

Figure 2. Tentative scheme relating storage and cooking oxidative processes in meat and the production/elimination of radical oxygen species (ROS). It is detailed (1) Oxidized compounds that can be generated in meat during storage and cooking. (2) Some intracellular mechanisms involved in ROS formation, oxidative damage, and possible elimination through the antioxidant machinery. (3) TMAO formation after choline and L-carnitine metabolism by the gut microbiota and subsequent oxidation in the liver. 4-HNE, 4-hydroxy-nonenal; AGEs, advanced glycation end-products; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; LOO•, lipid peroxyl radical; MDA, malondialdehyde; RAGE, receptor for AGEs- advanced glycation end-products; ROS, radical oxygen species; SOD, superoxide dismutase; TMA, trimethylamine; TMAO, trimethylamine N-Oxide.

4.1. Heme Iron

As previously commented, one of the first links in the oxidative chain processes is iron. This mineral is present in the diet as heme iron, derived from animal origin foods, and non-heme iron, in plant and animal origin sources [53]. Heme iron in meat is highly variable depending on the species, there being a greater amount in beef than in pork or chicken [8]. Heme iron constitutes myoglobin and hemoglobin, not being especially dangerous in oxidation terms. However, during the muscle into meat transformation, storage or digestion/metabolization, changes associated with oxygen depletion and decreased pH predispose heme iron to act as a pro-oxidant [42].

Iron presents the ability to alternate between oxidation states when accepting and donating electrons easily, changing from ferric (Fe^{3+}) to ferrous (Fe^{2+}) and vice versa (Figure 2). Given its oxidizing potential, we found that this mineral is normally bound to proteins for its storage and transport (forming complex with ferritin, transferrin or other proteins as hemoglobin, myoglobin, cytochrome c, cytochrome P450, nitric oxide synthases or guanylate cyclase) [54] that partially or almost completely blocked its oxidation. However, when heme iron is free it can cause oxidative damage since it rapidly loses the heme porphyrin ring and contributes in the ferrous state to ROS generation through Fenton reaction. A high heme iron intake can induce lipid peroxidation (LPO) in two main ways: in the initiation stage, catalyzing the ROS formation; or in the propagation phase,

catalyzing the peroxide decomposition to promote LPO. Heme iron catalyzes the superoxide and hydrogen peroxide conversion to hydroxyl radicals (OH•) by the Fenton reaction (Figure 2) [44]. Due to its lipophilic nature, heme toxicity is further exacerbated by its ability to insert itself into lipid membranes and to catalyze cell membrane oxidation, with the subsequent cytotoxic lipid peroxide formation which can lead to cell death [55]. It can also act as a nitrosating agent after being metabolized by intestinal bacteria and generate N-nitroso compounds, capable of causing DNA damage or DNA adducts formation [56]. In addition, heme iron can also participate in lipoprotein oxidation and lipid peroxidation end-products formation, such as MDA, 4-HNE, oxysterols, and aldehyde during meat storage, processing, and digestion [56].

Iron absorption and metabolism are tightly regulated in vivo in an attempt to prevent reactive iron species from participating in uncontrolled oxidation reactions [54,57]. Iron is absorbed by the enterocyte apical membrane in the form of heme iron or as Fe^{2+} and Fe^{3+} being rapidly metabolized for storage as ferritin or exported through ferroportin. An intracellular heme iron overload can affect the redox state, where the enzyme heme oxygenase (HO), especially the inducible form HO-1, plays an essential role in breaking it down to produce biliverdin, carbon monoxide, and Fe^{2+} [54,55]. Biliverdin is rapidly reduced by biliverdin reductase to produce bilirubin, which can effectively remove peroxyl radicals, thereby inhibiting LPO, attenuating heme-induced oxidative stress, cell activation, and death [55]. Likewise, the released carbon monoxide activates nuclear factor E2-related factor 2 (Nrf2) signaling, promoting the endogenous antioxidant availability and increasing HO-1 levels [58]. On the other hand, ROS stimulate ferritin synthesis and iron sequestration, being essential for proper iron homeostasis [55]. Therefore, heme iron can contribute to oxidative processes both in meat and in the organism once consumed. However, within healthy nutritional patterns, the endogenous antioxidant system is capable of alleviating and also blocking the oxidative effect induced. Again, several different situations made this topic complex and difficult to study and clearly demand to avoid doing any generalization as iron content in meat is rather variables as we have discussed in Section 4. In addition, the consumption of "red" meat is expected to induce more oxidation effects after a large interprandial period (e.g., 15 h) that after a shorter one (4–5 h) as the activity and expression of gastrointestinal antioxidant enzymes is highly modified by the fasting conditions [59].

We also have to keep in mind that most studies have been performed in raw meat or in in vitro model conditions, with no final conclusions on the importance of cooking and the type of cooking on meat oxidation. More, researches on the interaction of cooking–digestion oxidations are scarce [60]. When heated, Fe^{2+} is released by the heme-porphyrin moiety destruction, oxymyoglobin releases O_2, leading to H_2O_2 production, and antioxidant enzymes (such as glutathione peroxidase) are inactivated [60]. These reactions favor the stimulation of the Fenton reaction, which leads to greater LOP formation. On the other hand, several cooking methods can be applied to cook meat in order to increase its acceptability and digestion, which in turn modify original composition and give rise to oxidation. The effect of cooking on lipid oxidation has been related to the warmed-over flavor in cooked meats. The explanations offered take into account factors associated with the thermal treatment itself (cooking methods, final cooking temperature or cooking rate) and with the product composition (amount and type of lipid, antioxidant, etc.) [61]. In a previous study, meat lipid oxidation was evaluated by monitoring secondary oxidation compounds (thiobarbituric acid reactive substances—TBARS) [62]. When specific cooking procedure (conventional oven, microwave oven, electric grill, and pan-frying) were applied to same restructured beef meat formulated to present low or medium fat content the TBARS changed. However, results suggest that oxidation was low in all restructured steaks (Figure 3). Irrespective of product formulation, the cooking process did not always cause an increase in TBARS values; however, the most important changes in lipid oxidation induced by cooking were observed in low fat samples, mainly those cooked in a microwave oven (Figure 3A). Microwaving has been reported to induce increased thermaloxidation in oils [63]; however, this effect was not observed in medium fat steaks. Similar effects were observed in pan-frying and grilling [64]. Thus, in some way it

seems that the fat presence exerts some kind of protection against oxidation. It could be speculated that fat avoid the releasing of Fe^{2+} in low fat samples but this hypothesis was not evaluated.

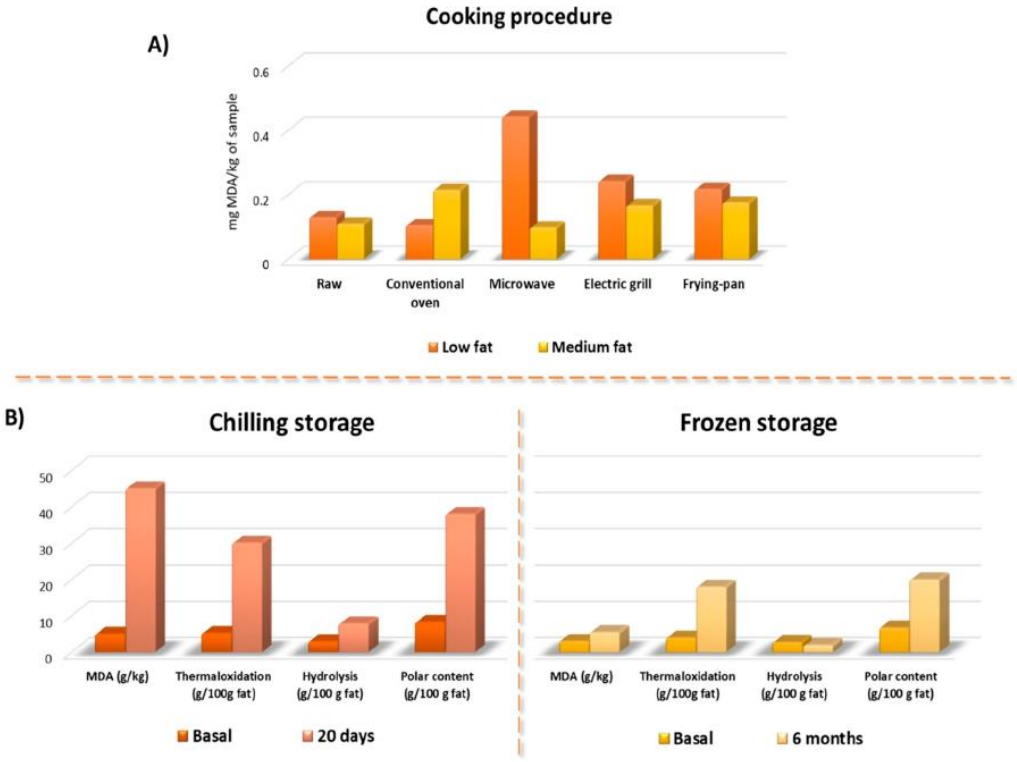

Figure 3. Oxidation compounds formation according to the cooking and storage method. (**A**) Results of the MDA formation in beef steaks with low and medium fat content subjected to different cooking methods. (**B**) Oxidation compounds formation in preserved meat in chilling or frozen storage. Modified from Serrano et al. [62] and Librelotto et al. [64]. With Meat Science permission solicited and still pending.

Recently we have published the importance of frying on Mediterranean cousin, technique that contrarily to that it is though permits to improve food fatty acid composition and stability depending on the oil type used [65]. In fact, seed oils are more susceptible of thermaloxidation that monounsaturated oils, determining that food cooked with seed oils contain higher amounts of these undesirable compounds [66], thus contributing, as in the specific case of Milanese, a typical Italian heritage-Mediterranean food, to some potential negative effects of meat consumption. Again, we think that several aspects are still pending to be more deeply studied.

4.2. Malondialdehyde and Polar Triglycerides

MDA is an organic compound resulting from the LPO of arachidonic acid and other long-chain PUFAs through enzymatic and non-enzymatic processes [67]. It is important to note that during meat processing and storage, the MDA formation takes place through non-enzymatic processes, derived from lipid peroxidation. However, in a living organism, the MDA formation can be produced both by a non-enzymatic mechanism and by enzymatic processes during the biosynthesis of thromboxane A 2 (TXA 2) and 12-l-hydroxy-5,8,10-heptadecatrienoic acid (HHT) [68]. MDA is one of the most abundant and well-known aldehydes generated during secondary lipid oxidation and is also the most widely used as an oxidation marker in meat and meat-products. Once consumed, MDA can be

enzymatically metabolized, or it can interact with proteins, nucleic acids and lipids, being able to alter a great variety of biological molecules [69]. Lipoproteins are also more susceptible to oxidation after red meat consumption, which is a major problem for CVD. It can establish covalent modifications within lipoproteins, especially with LDL, as observed in studies in humans where higher plasma MDA levels and oxidized-LDL after meat chops consumption were found in comparison to its turkey meat counterpart.

Current evidence suggests the presence of high plasma MDA levels after red meats or processed products consumption by healthy individuals [70]. This could be due, partially, to the fact that the stomach acts as a bioreactor, generating a large amount of ROS and promoting the LPO. Studies of in vitro digestions have demonstrated that the meat fatty profile is a determinant for the MDA formation in its passage through the gastrointestinal tract [49]. Although no clear explanation is available, high iron levels seem to contribute to oxidation at the stomach [71].

Polar material determination has proven to be one of the most specific methods to analyze fat and oil alterations [72]. The polar material content consists of glycerides and free fatty acids with higher polarity than those the original triglycerides, as polymers of triglycerides, dimers of triglycerides; oxidized triglycerides; diglycerides; monoglycerides; and free fatty acids. Based on our group large experience on evaluating fat oxidation by column chromatography followed by high-pressure size exclusion chromatography [72], the meat fat alteration was evaluated in restructured pork models submitted to chilling and frozen storage [10]. MDA was also determined by meaning of the TBARS analysis. TBARS, thermal oxidized compounds (polymers of triglycerides, dimers of triglycerides; oxidized triglycerides) as well as hydrolytic compounds (diglycerides; monoglycerides; and free fatty acids) increased significantly during storage. Several compounds affected oxidation in the tested meat systems (a) relatively high fat percentages, which generally promote oxidation susceptibility; (b) prepared with added NaCl, and salting that is known to increase the prooxidant activity of iron in myofibrillar foods; (c) non addition of some common additives with antioxidant activity (e.g., nitrites, ascorbic acids, etc.); (d) major reduction in particle size (and hence loss of structural integrity) which increased the exposure of labile compounds to oxygen; (e) heating during meat system preparation (70 °C) [10]. A detailed observation of results suggests that fat alteration after 6-month chilling was about the half that that observed after 20 days at chilling conditions (Figure 3B).

4.3. 4-Hydroxy-Nonenal

4-HNE is a secondary oxidation product from LPO and potentially has genotoxic properties [49]. 4-HNE has been extensively studied, not only for its function as a signaling molecule that stimulates gene expression, but also for its cytotoxic role that inhibits gene expression and promotes the development and progression of different pathologies. 4-HNE can be formed endogenously through enzymatic and non-enzymatic processes, although considerable levels have also been found in foods. Similar to MDA, the 4-HNE formation by enzymatic processes is due to the metabolism of ω-6 PUFA by 15-lipoxygenase. On the other hand, in the non-enzymatic formation of this oxidation product, five possible routes have been described, for which it is recommended to review Ayala et al. [68]. In meat-products, after interacting with its histidine residues, 4-HNE promotes myoglobin redox instability, accelerating meat discoloration and inducing LPO [73]. This covalent modification induces iron liberation from the pigment, facilitating further meat worsening [74]. 4-HNE is also known as a second oxidative stress messenger and has a longer half-life than ROS (Figure 2). Its possible involvement in numerous pathological processes such as metabolic diseases, neurodegenerative diseases, and cancers has been observed, probably due to its chemical reactivity and ability to form covalent adducts with biological molecules [68].

As positive association between heme iron levels and 4-HNE formation has been revealed in both animal diets and meat-products, it seems crucial to control the 4-HNE presence in the diet, mainly as meat-products or foods rich in ω-6 PUFA or selenium. Metabolism and the subsequent 4-HNE elimination is predominantly done through the glutathione system by reduced glutathione (GSH)

conjugation by glutathione-S-transferase to originate the conjugate GSH-4-HNE although there are also other alternative pathways involving aldehyde dehydrogenase and alcohol dehydrogenase enzymes. Once inactivated, 4-HNE is eliminated as conjugated metabolites through urine or bile (Figure 1) [75].

4.4. Oxysterols

Cholesterol is a noteworthy component of meat and meat-products. Given its lipid nature, it is susceptible to oxidation during the production and storage process, giving rise the dreaded oxysterols or cholesterol oxidation products, whose levels in fresh products are very low [76]. These oxidative modifications involve the addition of one or more oxygen-containing functional groups on the sterol B ring and/or on the side chain. Of the different oxysterols that can originate in food, the most common are 7-oxygenated sterols and 5α,6α-oxygenated sterols [77]. The average oxysterols amount present in meat and meat-products varies from 0.1 to 18.7 μg/g [76]. However, these concentrations may modify depending on the heat treatment, the type and duration of storage and the meat matrix composition [77,78]. The oxysterols formation is a temperature-dependent process, as dramatic increase in foods cooked to high temperatures was observed [79,80]. On the other hand, as with the vast majority of lipids, light and the oxygen presence are key factors in oxidative processes, both conditioned by exposure time [78]. Furthermore, all these processes are influenced by the lipid composition of the meat-matrix, with a greater formation of oxysterols being observed in PUFA-rich meat-products [76,81].

The presence of oxysterols in the body can be of endogenous origin (mainly derived from non-enzymatic processes) or exogenous, from the diet. Although oxysterol absorption rate is lower than that of cholesterol, there is a direct correlation between the amount of oxidized lipids ingested and plasma oxysterols levels [82,83]. These compounds are absorbed in the upper digestive tract and are transported by chylomicrons and other lipoproteins, being able to deposit them in peripheral tissues and increase oxidative stress. They are generally eliminated through bile, after metabolization by the liver (Figure 1) [77,84]. Various studies have suggested that oxysterols are potentially involved in the onset and progression of chronic diseases such as atherosclerosis or T2DM since they have proinflammatory, cytotoxic, and mutagenic properties [76,85].

4.5. Protein Carbonyls

In addition to lipid substrates oxidation, it is currently known that proteins in meat-products can be a source and target of ROS [86]. These alterations had gone unnoticed until recently when oxidized proteins were suggested to have a negative impact on meat quality [87,88]. Numerous studies reported that protein oxidation takes place during the post-mortem stage, meat handling, processing, and storage [86]. However, current evidence largely ignores how oxidative processes take place from proteins, peptides, and amino acids. What does seem evident is the interaction between lipids and oxidized proteins lead to a marked meat deterioration [89,90].

Protein oxidation results from a chain reaction similar to that occurring in lipids, in which superoxide, hydroperoxyl and hydroxyl radicals, hydrogen peroxide, hydroperoxides, peroxyl radicals, and heme iron seem to be the triggering factors. The level and nature of the oxidation products formed largely depends on the amino acids involved and how the oxidation process begins [86,91]. Among the possible modifications that may be suffered, the formation of protein carbonyls (aldehydes and ketones) is the most prominent and has become the most common measurement to evaluate protein oxidation in meat and biological systems because it is easily measurable through the 2,4-dinitrophenylhydrazine (DNPH) technique [92,93]. Carbonylation is an irreversible and non-enzymatic modification of proteins, which mainly takes place from the direct oxidation of the lysine, threonine, arginine, and proline side chains. Other possible oxidation routes have also been described, such as enzymatic non-glycation in the presence of reducing sugars, oxidative cleavage of the peptide skeleton through the α-amidation pathway or by oxidation of glutamyl side chains and covalent binding to compounds of non-protein carbonyl such as MDA or 4-HNE [86,94] whose origin and relevance has been already discussed in previous subsections.

Furthermore, studies have shown that oxidized components consumption in the diet increases oxidation markers in both blood and tissues, promoting oxidative stress. Despite the fact that most of the available literature has focused on the effect of oxidized lipids consumption, some studies indicated that oxidized protein intake increases oxidative stress both in blood and in the digestive tract of mice [95,96]. In line with these data, other authors already establish a relationship between its consumption and the development of chronic diseases such as T2DM [94,97,98].

4.6. Advanced Glycation End-Products

AGE products are compounds generated from a non-enzymatic reaction between reducing sugar and the amino groups of proteins, lipids, or nucleic acids, traditionally known as the Maillard reaction [99]. AGEs are highly oxidizing compounds that are also known as glycotoxins and can be formed endogenously or ingested through the diet [100]. These same authors have developed a database where they summarized the AGEs amount in food, with a special mention for meat and meat-products and suggest that due to its high consumption meat-products could highly contribute more to AGEs daily intake. Furthermore, the AGEs formation is notably increased by high temperatures cooking, one of the most common practices in meat preparation [100].

High consumption of AGEs has been related to CVD, kidney disease, and especially with T2DM [100–102]. AGEs present relatively high bioavailability as 10% of the consumed AGEs are absorbed in the intestine and contribute to the AGEs systemic levels and the formation of ROS and increase oxidative stress (Figures 1 and 2) [102]. Their pathological effects have been related to their ability to promote oxidative stress and proinflammatory cytokines production. In addition, AGEs can induce oxidative stress by interacting with the insulin receptor, whose union has been shown to increase ROS production through NADPH oxidase [103]. Moreover, it was shown that AGEs upregulate the receptor for AGEs (RAGE) expression in various cell types, entering a vicious circle and inducing the transcriptional factor nuclear factor-κB (NF-κB) sustained activation [101].

4.7. Trimethylamine N-Oxide

Currently, research on meat consumption focuses on the study of TMAO. This is an organic compound generated by intestinal bacteria from choline and L-carnitine rich sources. Fundamentally, choline is absorbed in the small intestine, but when there is excessive supply—as occurs after there has been a high red meat intake—, it reaches the large intestine and is metabolized by the microbiota to produce trimethylamine. Besides, L-carnitine undergoes bacterial processing similar to choline. In both cases, the trimethylamine formed oxidizes rapidly in the liver and gives rise to TMAO (Figure 2) [104]. Different metaanalyses have established a strong relationship between elevated circulating TMAO levels and CVD risk [105]. In fact, a high red meat intake increases the systemic TMAO levels in healthy humans, mainly due to the greater availability of dietary precursors, the TMAO bigger formation by the microbiota and the lower renal excretion [106]. Several studies have found that plasma TMAO levels can affect lipid homeostasis by modulating cholesterol metabolism [107]. Likewise, recent research has shown that TMAO can play an important role in endothelial dysfunction and in atherosclerosis development since it promotes oxidative stress by increasing ROS and MDA levels and reduces the superoxide dismutase (SOD) levels. Similarly, it also participates in vascular inflammation by inhibiting the expression and activity of eNOS, reducing nitric oxide production, increasing NF-κB signaling, inducing inflammasome activation and the release of proinflammatory cytokines [108–111].

5. Practical Implications and Future Works

Several scientific societies have called for the need to consume a plural diet in which both animal and vegetable foods should be present to diminish potential deleterious effects derived from subclinical deficiencies and bad storage and cooking practices. Apart from its nutritional composition, one of the major advantages of meat and meat-products is being a good matrix to include functional ingredients that, in addition to improving meat composition and health properties, they would contribute changing

the currently negative image attributed to this food-group [2,19–21]. Research and development of functional meat should take advantages of these previous premises in order to employ the most convenient meat, under a health point of view, to incorporate health ingredients. However, the need for strict controls of the ingredient quality used in functional meat design and preparation has to be pointed out in order to avoid the presence of undesirable contaminants or inadequate composition. As meat is normally storage at a low temperature before consumption and submitted later to different cooking treatments, active research is demanded to increase knowledge of reliable deleterious effects on oxidation.

As for other foods, the potential negative interactions with other dietary compounds (e.g., food, nutrients) have to be tested for regular and functional meats. The concern is focused on drugs that are metabolized by cytochrome P450 isoenzymes, as we have commented on, how several ingredients (e.g., polyphenols) affect this metabolizing enzyme highly modifying or even reversing the antioxidant properties of a given functional meat to a pro-oxidant one.

Although growing evidence exists, most obtained data come from in vitro studies being urgent to perform in vivo studies in animals and human. Individual response to therapeutic diets is striking which appears to have a genetic component [16]; thus, genetic factors (presence of risk allele) affecting dietary response to antioxidant ingredients of functional meat should be investigated in order to get more targeted and potentially efficient functional meats for the prevention or amelioration of oxidation and their deleterious consequences [111]. Therefore, the presence of alleles negatively contributing to a lower antioxidant activity of GR, GPx, NO syntase, parooxonase (PON-1) or to a higher prooxidant activity induced by cyclooxygenase, lipoxygenase and different isoforms of cytochrome P450 in some individuals should be investigated by meaning of GWAS and EPIWAS trying to get a GRS (Gene-risk score) that advances the understanding of the reality of nutrigenetics on functional foods addressed to improve antioxidant status [112]. As colonic microbiota highly contributed to systemic oxidation [113], the effects of functional foods on microbiota diversity, abundance, genomics characteristics (metagenomics) and antioxidant/pro-oxidant activities should be investigated, with the use of the new Omics modalities/sciences/strategies: transcriptome, proteome, metabolome, metagenome being necessary for a more precise design of functional meat and its application to precision diets. Finally, we want to highlight the importance of personalized nutrition, since, as suggested by Van Hecke et al., a high consumption of red and processed meat may present a higher risk for some population subgroups, for example, individuals infected with *Helicobacter pylori* those with inflammatory bowel disease, which are common gastrointestinal diseases associated with oxidative stress [8]. Likewise, the intestinal microbiota can also play a key role in the possible involvement of degenerative diseases associated with oxidative stress.

Animal studies have reported that eating processed meat promotes proteolytic bacteria growth associated with non-alcoholic fatty liver disease and obesity [114–116]. It is also known that a relatively high proportion of ingested heme iron reaches the colon, which could stimulate oxidation reactions in the colon, especially when large amounts of red meat are ingested [116]. In fact, the increase of oxidized lipids in colonic tissues is one of the proposed mechanisms that relate red meat consumption to colorectal cancer development [117,118]. All these processes are interconnected so that an excessive arrival of oxidized products derived from meat could induce intestinal dysbiosis, alter its metabolic capacity, and facilitate deleterious effects on colonic mucosae [119,120]. Some authors suggest the hypothesis that this relationship could be due to heme iron-induced dysbiosis since a greater presence of bacteria considered pathogenic (e.g., *Streptococcus bovis*, *Bacteroides*, *Enterococcus faecalis*, *Escherichia coli*, and *Clostridia*) in the presence of this mineral has been observed [121]. Well-controlled studies have to be performed both in animal models and humans to find out if all these processes are interconnected.

6. Conclusions

Meat and meat products are amply consumed all over the word, significantly contributing to people's nutrition.

Low to very low meat consumption makes it highly difficult to cover dietary recommendations, particularly of vitamins B_6 and B_{12}.

Several cuts (pieces) of terrestrial and marine mammals, differing in species, gender, age, livestock practices, and feeding, are included in the concept of meat.

There are large dissimilarities (e.g., fat infiltration, fatty acids, and fat-soluble vitamins content and composition) between meat from monogastric and polygastric animals.

Their consumption highly exceeds the daily amount considered adequate (mainly due to the high SFA and heme iron contents) and because of this, high intake induces the low consumption of other food-groups conditioning health status.

At present, no consensus on the concept and differences between red and white meat is available.

Iron and PUFA meat contents highly contribute to meat oxidation during storage and cooking.

Meat and meat-products oxidation give rise to increased MDA and polar material, 4-HNE, oxysterols, protein carbonyls, AGEs, and TMAO levels contributing to the deleterious effects ascribed to high meat consumption.

Meat and meat-products digestion and metabolization contribute to body oxidation status.

Long-term intervention-controlled studies testing different types and amounts of meat and meat-products are demanded to accurately ascertain the relationship between meat, oxidation, and degenerative diseases.

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Abbreviations

4-HHE: 4-hydroxy-hexenal; 4-HNE, 4-hydroxy-nonenal; AGEs, advanced glycation end-products; CAT, catalase; CVD, cardiovascular diseases; DNPH, 2,4-dinitrophenylhydrazine; GPx, glutathione peroxidase; GR, glutathione reductase; GRS, gene-risk score; GSH, reduced glutathione; GSSG, oxidized glutathione; HDL, high density lipoproteins; HO, heme oxygenase; LDL, low-density lipoproteins; LPO, lipid peroxidation; MDA, malondialdehyde; MUFA, monounsaturated fatty acids; NF-κB, factor nuclear factor-κB; Nrf2, nuclear factor E2-related factor 2; PUFA, polyunsaturated fatty acids; PON-1, paraoxonase-1; RAGE, receptor for AGEs- advanced glycation end-products; ROS, reactive oxygen species; SFA, saturated fatty acids; SOD, superoxide dismutase; T2DM, type 2 diabetes mellitus; TBARS, thiobarbituric acid reactive substances; TMAO, trimethylamine N-Oxide; VLDL, very low density lipoproteins.

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Publicación 5

Functional Meat Products as Oxidative Stress Modulators: A Review

Macho-González A, Bastida S, Garcimartín A, López-Oliva ME, González P, Benedí J, González-Muñoz MJ & Sánchez-Muniz FJ

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Resumen: Un elevado consumo de carne y productos cárnicos se ha asociado positivamente con un incremento del estrés oxidativo y el desarrollo de patologías crónico-degenerativas (Artículo 4). La carne y los productos cárnicos son una excelente matriz para incorporar compuestos bioactivos con potencial antioxidante. Como consecuencia, en la presente revisión evaluamos los principales ingredientes con efectos antioxidantes y si su empleo en el diseño y formulación de cárnicos funcionales puede ser una estrategia adecuada para mejorar la composición y estabilidad de la carne, así como paliar los efectos oxidativos atribuidos a su alto consumo.

Por ello, este trabajo analiza y resume: a) el concepto de alimento funcional, a la vez que se describen los principales procesos tecnológicos que permiten su formulación; b) principales trabajos que estudian el potencial antioxidante de productos cárnicos funcionales durante su procesado, almacenamiento y cocinado; c) resultados de ensayos con animales y estudios de intervención en humanos, tratando de determinar la funcionalidad del producto final (p. ej. neutralizando la formación radicales libres o aumentando la maquinaria antioxidante); d) los posibles efectos negativos de un consumo elevado de cárnicos funcionales; e) la aplicación de los cárnicos funcionales en la nutrición personalizada o de precisión, identificando aquellos sujetos que se podrían beneficiar en mayor medida de su consumo.

El desarrollo de este tipo de productos presenta por tanto un enfoque doble, ya que por un lado pueden reducir el estado de oxidación de la carne y prolongar su vida útil, y por otro, mejorar el estatus antioxidante de los consumidores, contribuyendo a la prevención y tratamiento de enfermedades en las que se produce un elevado estrés oxidativo.

REVIEW

Functional Meat Products as Oxidative Stress Modulators: A Review

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ABSTRACT

High meat consumption has been associated with increased oxidative stress mainly due to the generation of oxidized compounds in the body, such as malondialdehyde, 4-hydroxy-nonenal, oxysterols, or protein carbonyls, which can induce oxidative damage. Meat products are excellent matrices for introducing different bioactive compounds, to obtain functional meat products aimed at minimizing the pro-oxidant effects associated with high meat consumption. Therefore, this review aims to summarize the concept and preparation of healthy and functional meat, which could benefit antioxidant status. Likewise, the key strategies regarding meat production and storage as well as ingredients used (e.g., minerals, polyphenols, fatty acids, walnuts) for developing these functional meats are detailed. Although most effort has been made to reduce the oxidation status of meat, newly emerging approaches also aim to improve the oxidation status of consumers of meat products. Thus, we will delve into the relation between functional meats and their health effects on consumers. In this review, animal trials and intervention studies are discussed, ascertaining the extent of functional meat products' properties (e.g., neutralizing reactive oxygen species formation and increasing the antioxidant response). The effects of functional meat products in the frame of diet–gene interactions are analyzed to *1*) discover target subjects that would benefit from their consumption, and *2*) understand the molecular mechanisms that ensure precision in the prevention and treatment of diseases, where high oxidative stress takes place. Long-term intervention-controlled studies, testing different types and amounts of functional meat, are also necessary to ascertain their positive impact on degenerative diseases. *Adv Nutr* 2021;00:1–26.

Keywords: functional meat, bioactive ingredients, metabolism, oxidation status, antioxidant mechanisms, animal trials, intervention studies, precision diet

Introduction

Although meat and meat products are essential foods for most populations worldwide, high meat and meat-product consumption can induce pro-oxidant status in consumers (1). These foods can be a source of oxidized compounds (given their highly perishable nature), which are generated during processing, storage, cooking, digestion, and metabolism (2). Therefore, high meat consumption increases the risk of developing diseases associated with oxidative stress such as obesity, type 2 diabetes mellitus (T2DM), colorectal cancer, and cardiovascular diseases (CVDs) (1–4).

Nonetheless, because the worldwide consumption of meat and meat products is very high, it seems plausible to use them as matrices to ensure adequate consumption of bioactive ingredients (5). Moreover, besides extending the meat product's shelf-life (by minimizing meat oxidation) and improving its composition (by incorporating antioxidant molecules), it might also benefit health by reducing the body's oxidative status (2, 6). There is increasing interest in the search for healthier foods that can provide benefits beyond the merely nutritional (5, 7, 8). In this review, we assess if designing and consuming functional meat products is a suitable strategy to improve meat composition and stability,

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Abbreviations used: AE, arylesterase; CAT, catalase; COX, cyclooxygenase; CVD, cardiovascular disease; CYP7A1, cytochrome P450 7A1; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; LPO, lipid peroxidation; LTB4, leukotriene B4; MDA, malondialdehyde; NAFLD, nonalcoholic fatty liver disease; Nrf2, nuclear factor E2-related factor 2; OVN, optimum vitamin nutrition; oxLDL, oxidized LDL; PON1, paraoxonase-1; ROS, reactive oxygen species; SOD, superoxide dismutase; T2DM, type 2 diabetes mellitus; TXB2, thromboxane B2; 4-HNE, 4-hydroxy-nonenal.

Publicación 6

Papel de las proantocianidinas sobre la microbiota, permeabilidad intestinal e inflamación

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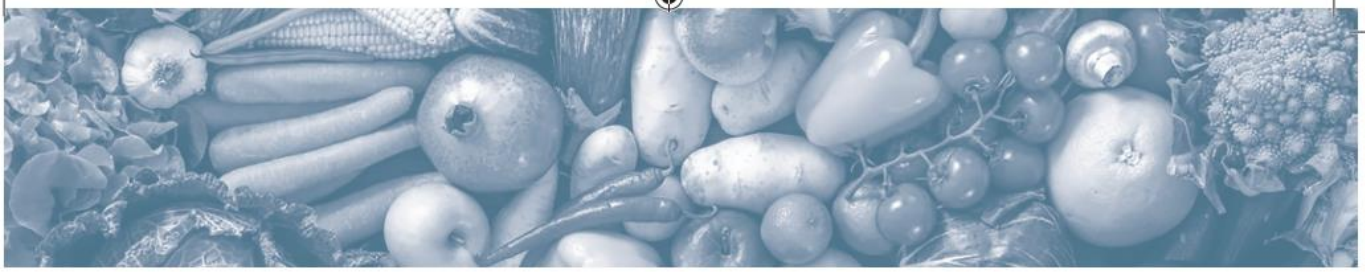
Resumen: Las proantocianidinas (PACs) o taninos condensados son unos compuestos polifenólicos que, dada su estructura básica conformada por unidades de flavan-3-ol, pertenecen al grupo de los flavanoles. Se encuentran ampliamente distribuidos en alimentos de origen vegetal como el té, café, arándanos o algarroba, cuyo consumo se ha relacionado con numerosas propiedades beneficiosas. Sin embargo, las PACs son estructuras complejas que necesitan un proceso de metabolización intestinal para ser biodisponibles, jugando un papel esencial en la modulación de la microbiota. En este capítulo de libro se revisa a) proceso de biotransformación de las PACs y su impacto sobre la microbiota intestinal tanto en una situación fisiológica como patológica; b) posible implicación de las PACs sobre la homeostasis del ecosistema digestivo, teniendo en cuenta el equilibrio entre la microbiota, la producción de mediadores metabólicos, la permeabilidad intestinal y la inmunidad local; c) otros efectos menos conocidos de las PACs sobre el tracto digestivo superior (cavidad bucal, gástrica e intestinal); d) efecto del consumo de PACs sobre diversas patologías crónicas como la aterosclerosis, hipertensión, dislipemia, obesidad, síndrome metabólico y DMT2; y por último, e) futuras líneas de actuación para poder establecer pautas dietéticas personalizadas en las que se incluyan PA para la prevención y tratamiento de las principales patologías crónica-degenerativas.



Papel de las proantocianidinas sobre la microbiota, permeabilidad intestinal e inflamación

16

Adrián Macho-González, Alba Garcimartín, Elvira López-Oliva, Juana Benedí, Sara Bastida, Francisco J. Sánchez-Muniz*

RESUMEN

Las proantocianidinas (PA), también denominadas taninos condensados, son compuestos polifenólicos vegetales que pertenecen a la subcategoría de flavanoles. Constituyen el grupo más abundante de fenoles naturales de la dieta occidental. Las PA difieren en el grado de polimerización y estructura, lo que a su vez condiciona su metabolización y sus efectos. En este capítulo se revisan aspectos de la biotransformación de las PA con actuación de la microbiota colónica tanto en modelos experimentales como en humanos, ambos en situaciones fisiológicas o patológicas, y las acciones de las PA sobre la pluralidad de géneros bacterianos que componen la microbiota. A su vez, se revisan las acciones de las PA a nivel local de la barrera intestinal (p. ej. producción de mucina, morfología de la mucosa, producción de IgA secretora, etc.) y sistémico, sobre marcadores de inflamación y perfil leucocitario. Por último se revisan algunas publicaciones de enfermedades crónicas (p. ej. aterosclerosis, obesidad, síndrome metabólico) donde se ha observado efecto de las PA en el patrón inflamatorio de bajo grado existente y por ende sobre el perfil de células inmunes. En este capítulo se incluye información preliminar de algunos estudios realizados por nuestro grupo con cárnicos funcionales que contienen extractos de algarrobo ricos en PA. El capítulo termina proponiendo futuras pautas de actuación, particularmente en humanos, en relación con la interacción de las PA y fármacos de uso común en patologías crónicas y en la necesidad de estudiar aspectos nutrigenéticos que explique diferencias interindividuales a la dietoterapia con PA, a fin de conocer la población diana que más se beneficiaría del consumo de PA.

INTRODUCCIÓN

Las proantocianidinas (PA), también denominadas taninos condensados, son compuestos polifenólicos vegetales que pertenecen al grupo de los flavonoides y, dentro de éste, a la subcategoría de flavanoles. Son el grupo más abundante de fenoles naturales después de las ligninas y son los flavonoides más abundantes de la dieta occidental. Son los responsables, en gran medida, de la textura, color y sabor astringente y amargo de algunos alimentos, como té, café, cacao, uva o algarrobo.[1] En los últimos años se está dedicando más atención a las PA, debido a la multitud de propiedades saludables que se atribuyen a su consumo, aspecto que ha motivado el diseño y comercialización de alimentos funcionales que las incorporan en su composición.[2] Durante varias décadas se han realizado numerosos estudios que demuestran su actividad antioxidante, antimicrobiana, anticancerígena, vasodilatadora y antiinflamatoria, resultando por ello beneficioso su consumo en patologías crónicas, fundamentalmente, las asociadas al síndrome metabólico (SM).[3] Dentro

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Capítulo 3

Evaluación del efecto antidiabético de un cárnico funcional enriquecido en extracto de algarroba sobre dos modelos murinos de DMT2

Antecedentes: la modificación del estilo de vida, con una alimentación saludable y mayor ejercicio físico, es la principal opción de prevención y manejo para la DMT2, aunque supone un gran reto para este grupo poblacional (American Diabetes Association, 2020; Bennett y cols., 2015). Como alternativa se están desarrollando cárnicos funcionales para mejorar el estado de salud sin la necesidad de cambiar excesivamente la alimentación. La carne es un alimento de amplia aceptación para todos los grupos poblacionales y una matriz excelente para incorporar compuestos bioactivos (Celada y cols., 2016; Sánchez-Muniz, 2004). Teniendo en cuenta los resultados descritos en el capítulo 1 sobre el metabolismo de los hidratos de carbono y las grasas y que predisponen al CFE como un prometedor agente antidiabético, seleccionamos una dosis de CFE adecuada para ser utilizada como ingrediente bioactivo en el desarrollo de un cárnico funcional dirigido a la población con DMT2. Para ello, diseñamos dos modelos murinos de DMT2 que simulan una etapa inicial y una etapa tardía de la patología. Asimismo, tal y como se verá a continuación en la descripción del diseño experimental, establecimos dos grupos de animales para ver la efectividad de la inclusión del cárnico funcional como tratamiento preventivo o terapéutico.

Como se ha comentado a lo largo de esta Tesis Doctoral, es importante evaluar que el consumo de un alimento funcional ejerce una acción beneficiosa en el organismo, más allá de la meramente nutricional. A pesar de ello, en la bibliografía disponible apenas hay estudios que evalúen su funcionalidad.

Objetivos:

- Elaboración de un cárnico funcional enriquecido en CFE por el método de adición de nuevos componentes; búsqueda de marcadores útiles para probar su eficacia.
- Evaluar el potencial antidiabético del consumo de un cárnico funcional enriquecido en CFE sobre la glucemia, lipemia y colesterolemia de ratas Wistar con DMT2.
- Estudiar el efecto del consumo de un cárnico funcional enriquecido en CFE sobre el metabolismo lipoproteico, la actividad arilesterasa y la oxidación

de las VLDL en dos modelos murinos de DMT2 (estadio inicial y tardío de la patología).

- Analizar el impacto del consumo de un cárnico funcional enriquecido en CFE sobre la señalización de insulina y la lipogénesis *de novo* hepáticas en dos modelos murinos de DMT2 (estadio inicial y tardío de la patología).
- Conocer el efecto del consumo de un cárnico funcional enriquecido en CFE sobre la microbiota, producción de AGCC y permeabilidad de la barrera colónica de ratas Wistar con DMT2 en estadio avanzado.
- Caracterizar el tipo de PACs presentes en el CFE.
- Evaluar el efecto del consumo de un cárnico funcional enriquecido en CFE sobre el estatus antioxidante de la mucosa colónica proximal y distal en ratas Wistar con DMT2 en estadio tardío.

Publicación 7

Carob-fruit-extract-enriched meat modulates lipoprotein metabolism and insulin signaling in diabetic rats induced by high-saturated-fat diet

Macho-González A, Garcimartín A, López-Oliva ME, Celada P, Bastida S, Benedí J & Sánchez-Muniz FJ

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29 de 89 en Nutrition & Dietetics

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Antecedentes: La RI es la alteración principal que define a la DMT2. A su vez, también es la causa endógena más frecuente del metabolismo de los lípidos y que contribuye al mayor riesgo de ECV encontrado en estos pacientes (Leavens & Birnbaum, 2011). La interrelación entre RI y la homeostasis lipoproteica parece estar en la ruta principal de señalización de la insulina hepática, la vía de InsR/IRS/PI3K/AKT. Se ha demostrado que su alteración influye en el ensamblaje de las VLDL y la cinética de las LDL a través de una menor expresión de su receptor, por lo que mejorar la RI parece ser una buena estrategia para manejar la dislipemia diabética (Verges, 2015). Las PACs de diferentes fuentes vegetales se han postulado como unos compuestos bioactivos con capacidad para modular la señalización de insulina y el metabolismo de los hidratos de carbono y las grasas (Rauf y cols., 2019). Por tanto, la inclusión de CFE en un reestructurado cárnico puede inducir una mejora en la RI y las alteraciones asociadas.

Hipótesis: La inclusión de CFE en una matriz cárnica reduce la dislipidemia diabética al mejorar la RI en ratas diabéticas alimentadas con dieta alta en grasas saturadas.

Resultados: Las dietas experimentales fueron bien aceptadas, aunque se observó una menor tasa de crecimiento en los animales CE, derivado de una menor ingesta y digestibilidad de la dieta, posiblemente por el efecto saciante de la fibra presente en el CFE. El consumo del cárnico enriquecido en CFE mostró un claro efecto antidiabético al reducir la insulinemia, HOMA-IR, lípidos plasmáticos e índice aterogénico. Esta reducción de la trigliceridemia, indujo una reducción del 36% de la masa total de VLDL y con un menor contenido en triglicéridos respecto al grupo C. Unido a esto, el grupo CE reveló un aumento de IDL y LDL, que junto con los niveles incrementados del receptor LDL, es indicativo de un mayor catabolismo de estas lipoproteínas. Todas estas mejoras en el metabolismo de las lipoproteínas podrían estar debidas, en parte, a una mayor efectividad en la señalización de insulina, ya que se observaron mayores niveles de PI3K y $pAKT^{ser473}$ en las ratas CE *vs*. C.

Conclusiones: La inclusión de un cárnico enriquecido en CFE mejora el metabolismo de las lipoproteínas al incrementar los niveles de algunas proteínas clave en la ruta InsR/IRS/PI3K/AKT de señalización de la insulina. Por lo tanto, se puede sugerir que la carne enriquecida en CFE es un alimento funcional adecuado para ser consumido por pacientes con DMT2 y ayudarles a mejorar la RI y la dislipemia.

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Carob-fruit-extract-enriched meat modulates lipoprotein metabolism and insulin signaling in diabetic rats induced by high-saturated-fat diet

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ABSTRACT

Insulin resistance (IR) produces qualitative, quantitative and kinetic changes of lipoproteins in Type 2 Diabetes Mellitus (T2DM). Carob-fruit-extract (CFE) has demonstrated antidiabetic properties but barely studies have evaluated the effect of CFE functional-meat consumption on early stages of T2DM. The current study was designed to analyze the effect of CFE-enriched meat-consumption on lipemia, lipoprotein profile and its relation with IR in T2DM model. Sixteen Wistar rats were divided in: C group, fed high-saturated-fat diet with commercial meat; and CE group, fed high-saturated-fat diet containing CFE-enriched meat. After 8-weeks, C rats showed hyperglycemia, hyperinsulinemia, hypertriglyceridemia and increased triglyceride-enriched-VLDL compared to CE animals ($p < 0.05$). CE group was able to partially block diabetic dyslipidemia and reduce HOMA-IR by showing higher PI3K, $pAKT^{ser473}$, GLUT2 and LDL-receptor levels *versus* their C counterparts ($p \leq 0.020$). In conclusion, CFE-enriched meat-consumption strongly counterbalanced diabetic dyslipidemia by improving insulin signaling, suggesting its being an adequate functional ingredient for T2DM.

1. Introduction

Type 2 Diabetes Mellitus (T2DM) prevalence has continued increasing in recent years, implying great clinical and social impacts. However, some pathophysiological facts remain poorly understood (Forouhi & Wareham, 2014). Insulin-resistance (IR) is the most frequent endogenous cause of lipid metabolism disorder contributing to higher cardiovascular disease (CVD) risk in T2DM patients (Laakso & Kuusisto, 2014). This pathology produces alterations in plasma lipids levels and consequently in lipoprotein metabolism (Verges, 2015). Fundamentally, T2DM presents quantitative, qualitative and kinetic lipoprotein alterations, which are clearly influenced by IR. The quantitative abnormalities consist in chylomicrons and VLDL increase, as a consequence of hypertriglyceridemia, and plasma HDL-level reduction (Parhofer, 2011; Tomkin & Owens, 2017; Verges, 2015). On the other hand, among qualitative changes, the most relevant are the presence of large VLDL particles, small and dense LDL, and triglyceride-enriched HDL. Due to alterations in chylomicrons, VLDL and LDL catabolism, kinetic changes can also be observed, which contributing to increase CVD risk (Mbue, Mbue, & Anderson, 2017; Parhofer, 2011; Tomkin & Owens, 2017). All these T2DM modifications seem to be related to hepatic insulin signaling alterations. The insulin receptor (InsR)/phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which is highly involved in the metabolic actions of insulin, is altered in IR (Leavens & Birnbaum, 2011; Tangvarasittichai, 2015). It has been demonstrated that disturbances in this pathway influence liver VLDL assembly and lipoprotein kinetic as a result, among other factors, of LDL-receptor (LDLr) expression reduction. Therefore, achieving better IR seems to be

Abbreviations: AE, arylesterase; AI, atherogenic index; CVD, cardiovascular disease; CFE, carob fruit extract; GSK3, glycogen synthase kinase 3; HDL, high-density lipoprotein; HSF, high-saturated-fat; IDL, intermediate-density lipoproteins; InsR, Insulin receptor; LDL, low-density lipoproteins; LDLr, LDL receptor; MUFA, monounsaturated fatty acid; PI3K, phosphatidylinositol 3-kinase; PON1, paraoxonase 1; PUFA, Polyunsaturated fatty acids; RM, restructured meat; SFA, saturated fatty acids; T2DM, Type 2 Diabetes Mellitus; VLDL, very low-density lipoproteins

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[1] These two authors should be considered the first author of the paper due to their equivalent contribution to the paper.

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an adequate therapeutic strategy to manage dyslipidemia in T2DM (Tomkin & Owens, 2017; Verges, 2015).

At present, a direct relationship between meat intake, especially red meat, and T2DM-risk has been suggested (Schwingshackl et al., 2017). Other studies support ensuring protein-consumption to maintain or improve insulin secretion in T2DM patients (Tian et al., 2017). Our group research has been working for decades on introducing bioactive compounds into meat matrix with the objective of reducing the potential negative effects attributed to meat consumption (Garcimartín, Santos-López, Bastida, Benedí, & Sánchez-Muniz, 2015; Olivero-David et al., 2011; Santos-López et al., 2018). Recent studies have demonstrated beneficial effects of fiber and polyphenols on carbohydrates and fats metabolisms and are highly recommended for the diabetic population (McRae, 2018; Xiao & Hogger, 2015). Taking this into account, we have developed a functional meat incorporating carob fruit extract (CFE). The consumption of this new meat product should counterbalance the low intake of fiber and polyphenols observed in diabetic patients (Kaline, Bornstein, Bergmann, Hauner, & Schwarz, 2007; McRae, 2018). CFE is a great source of insoluble fiber and proanthocyanidins, to which has been related to powerful antidiabetic properties. Specifically, we have already reported a reduction in both postprandial glycemia and lipemia in healthy rats fed CFE (Macho-González et al., 2017, 2018). Likewise, we have also observed a lipoprotein profile improvement after CFE-enriched meat consumption in later-stage T2DM rat model (Macho-González et al., 2019). Due to the scarcity of studies evaluating the effect of functional meat-consumption in the early stages of T2DM, we have tested the effect of CFE-enriched meat-consumption on an established rat model (Fang, Lin, Huang, & Chuang, 2019; Sah, Singh, Choudhary, & Kumar, 2016), in which this early stage of T2DM was induced by a high-saturated-fat (HSF) diet.

Therefore, we hypothesized that CFE inclusion into a meat matrix reduces dyslipidemia and IR in diabetic rats fed the HSF diet. The aims of this study were to analyze the effect of CFE-enriched meat consumption in diabetic rats induced by HSF diet on, a) the qualitative, quantitative and kinetic lipoprotein changes; b) the InsR/PI3K/AKT pathway modifications; and c) the relationship between lipoproteins, main insulin pathway markers, and IR changes.

2. Materials and methods

2.1. Carob fruit extract (CFE)

CFE is a natural product with 71–81% of insoluble dietary fiber, that contains 34–40% of high molecular weight proanthocyanidins, according to Patent WO2006/000551 (Ruíz-Roso, Requejo, Pérez-Olleros, & Holguin, 2006). This extract was obtained from the carob pulp following the procedure described in Patent WO2004/014150 (Ruíz-Roso, Requejo, Pérez-Olleros, Martín-Casero, & Haber, 2004) and Macho-González et al. (2018).

2.2. Restructured meat, diet preparation and experimental design

Diets and restructured meat (RM) were formulated according to the protocol described by Macho-González et al. (Macho-González et al., 2019) (Supplementary Table S1). Briefly, for each diet kilogram, 30% of RM and 70% of a purified formulated diet were mixed and subsequently sieved 3 times until a completely homogenous powder was obtained. Two experimental semisynthetic diets were prepared: a) containing a control-RM (C) and b) containing CFE-enriched meat (CE), whose composition is detailed in Table 1. Both diets contained 49.42%, 36.43 and 14.15% of the total Kcal from fats, carbohydrates and proteins, respectively. Diets were calculated to accomplish micronutrient requirements at their final concentration.

Two-month old male Wistar rats were obtained from the Harlan Laboratories models (Harlan S.L., Barcelona, Spain) and housed in couples under controlled temperature (22.3 ± 1.9 °C) and light (12-h light/dark cycle) in the Animal Experimentation Center of the Alcalá University, Madrid, Spain (register no. ES280050001165). Tap water and food were provided *ad libitum*. Two rat groups of 8 rats each were distributed according to the diet, C and CE. Each group received the same diet for 8 weeks. Each group received the same diet for 8 weeks. Food consumption was measured daily and body weight once a week. Feces were collected the last week and fecal fat was extracted and quantified as previously reported (Macho-González et al., 2018). In order to avoid inter-assay variations, overnight fasted rats were taken, anesthetized with isoflurane (5% v/v) and euthanized. Blood was collected from the descending aorta with a heparinized syringe in cold tubes and placed in ice until processing. Livers were removed, weighed, and prepared properly.

This study was approved by the Spanish Science and Technology Advisory Committee (project AGL2014-53207-C2-2-R) and by the Ethics Committee of the Complutense University of Madrid (Spain). Experiments were performed in compliance with Directive 86/609/EEC of 24 November 1986 (amended by EU Directive 2010/63/EU) on the protection of scientific research animals.

Table 1
Composition of the Restructured Meat (RM) incorporated to the experimental diets fed male Wistar rats.

Restructured meat components	Control-RM	CFE-RM
Protein, %	13.1	13.1
Fat, %	38.3	38.3
Water, %	46.9	46.9
Cholesterol, g/kg	0.74	0.74
SFA/MUFA/PUFA ratio	41.2/43.5/8.5	41.2/43.5/8.5
Ingredients		
Lean pork, g/kg	663.1	663.1
Lard, g/kg	331.1	331.1
Na, g/kg	0.5	0.5
STP, g/kg	0.1	0.1
Sodium nitrite, g/kg	1.2	1.2
CFE, g/kg	0.0	4
Cellulose	4	0.0

Control Restructured Meat (Control-RM), CFE-enriched meat (CFE-RM). STP, sodium tripolyphosphate. SFA, saturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, Polyunsaturated fatty acids.

2.3. Glycemia and insulin determinations

Blood samples were collected in fasting conditions in heparinized tubes at the middle of the third week and at the end of the experiment. Plasma was isolated by centrifugation for 10 min at 986g, and glycemia was quantified immediately in a plate reader (SPECTROstar Nano, BMG LABTECH, Offenburg, Germany) at 492 nm, using the GOD kit (Spinreact, Barcelona, Spain) (Macho-González et al., 2017). Insulin was measured using ELISA kit (Rat insulin ELISA Kit, ELR-Insulin, RayBiotech, Inc., USA) according to the manufacturer's manual. The colour intensity was evaluated at 450 nm using a microplate reader (SPECTROstar Nano). QUICKI index was calculated according to the formula 1/[(log insulin)(mIU/l) + (log glucose) mg/dl)] and HOMA-IR was calculated as: glucose (mmol/l) × insulin (mIU/l)/22.5 (Bowe et al., 2014; Gesteiro, Rodríguez Bernal, Bastida, & Sánchez-Muniz, 2012).

2.4. Lipoprotein isolation

Plasma was separated from the whole blood by centrifugation for 20 min at 615g and kept at 4 °C until lipoprotein isolation. A SW 40.1 rotor was used to separate the different lipoprotein fractions in 2 mL plasma samples by saline gradient ultracentrifugation during 21 h 40 min at 272,000g (40,000 rpm) at 4 °C (Beckman L8-70 M, California, USA) following a modification of the Terpstra et al. method (Terpstra,

Woodward, & Sánchez-Muniz, 1981) as previously described (Garcimartín et al., 2015). Lipoproteins were isolated according to conventional density range for rats [VLDL ($\rho_{20} < 1.0063$ g/mL), IDL ($1.0063 < \rho_{20} < 1.019$), LDL ($1.019 < \rho_{20} < 1.057$), and HDL ($1.057 < \rho_{20} < 1.21$ g/mL)] (Bocanegra et al., 2009).

2.5. Plasma lipid analysis and lipoprotein composition

Total cholesterol, triglycerides and phospholipids were determined in plasma and lipoprotein fractions (VLDL, IDL, LDL, and HDL) using standard enzymatic colorimetric tests (Spinreact) according to the manufacturer's manual in a plate reader at 492 nm (SPECTROstar Nano). Total lipids were calculated as the sum of total cholesterol, triglycerides, and phospholipids. The protein content of each lipoprotein fraction was analyzed by the Bradford method (Bradford, 1976). The total mass of each lipoprotein fraction was calculated as the sum of proteins and total lipids (cholesterol + triglycerides + phospholipids) (both in mg/dL) (Macho-González et al., 2019). The atherogenic index (AI) was calculated as described by Garcimartín et al. (Garcimartín et al., 2015).

2.6. Liver and VLDL oxidation (TBARS assay)

The Mihara and Uchiyama method (Uchiyama & Mihara, 1978) was used to quantify liver and VLDL oxidation (liver-ox and VLDL-ox, respectively). Malondialdehyde (MDA) formed after adding thiobarbituric acid to VLDL samples and liver extracts were measured in a fluorescence plate reader (FLUOstar Omega, BMG LABTECH, Offenburg, Germany) at 485/20 nm excitation and 528/20 nm emission wavelengths. MDA standard curve was performed to quantify liver and VLDL oxidations. Results are expressed as mg MDA mg/L of plasma for VLDL-ox and MDA/mg protein for liver-ox.

2.7. Arylesterase activity measurement

Plasma arylesterase (AE) activity was measured using simulated body fluid as a buffer and phenylacetate as a substrate according to Nus, Sánchez-Muniz, Gago, López-Oliva, and Sánchez-Montero (2008). Reaction rates of AE were followed at 270 nm in thermostatically controlled 10-mm Lightpath quartz cuvettes using a spectrophotometer (SPECTROstar Nano). Liver AE activity was measured in liver extracts according to Macho-González et al. (Macho-González et al., 2019) and results expressed in AE units/mg protein. One unit of AE was defined as the phenol (mmol) formed from phenylacetate per minute.

2.8. Western blotting

Immunoblots were performed using liver lysates. Protein extracts (DC Protein Assay Kit, Bio-Rad, Madrid, Spain) from each sample were separated by SDS–PAGE and transferred to polyvinylidene fluoride membranes (GE Healthcare, Madrid, Spain). Membranes were incubated overnight at 4 °C with the following primary antibodies: anti-LDLr, anti-insulin receptor β, anti-PI3K, anti-AKT2, anti-pAKT^{ser473}, anti-GSK3β, anti-pGSK3β^{ser9} and anti-GLUT2 (Santa Cruz Biotechnology, Quimigen, Madrid, Spain). Anti-β-actin and anti-α-tubulin were used as the loading control. Antibodies hybridization was revealed by incubating the membranes with the appropriate secondary antibodies conjugated with peroxidase for 1 h at room temperature. The chemiluminescence signal of membranes was read in an ImageQuant LAS 500 (GE Healthcare, Madrid, Spain) using the ECL kit Select-kit (GE Healthcare, Madrid, Spain).

2.9. Immunohistochemical staining

Paraffin-embedded liver sections were deparaffinized and rehydrated in a graded ethanol series. After retrieving citrate antigen and quenching endogenous peroxidase, sections were incubated with different primary antibodies overnight at 4 °C to determine the presence of: anti-LDLr, anti-insulin receptor β, anti-PI3K, anti-AKT2, anti-pAKT^{ser473}, anti-GSK3β and anti-pGSK3β^{ser9} (Santa Cruz Biotechnology). The color reaction was developed with a polymerized horseradish peroxidase-conjugated secondary antibody and counterstained with hematoxylin. The intensity of immunostaining for each antibody was measured using ImageJ free software (U. S. National Institutes of Health, Bethesda, USA). A total of 15 fields per section per rat (200× magnification for image analysis) were selected and analyzed. The positive staining intensity was calculated as percentage of the ratio of the stained area to the total field assessed.

2.10. Statistical analysis

Results were expressed as mean ± standard deviation (SD). Group results were compared by the unpaired Student's *t*-test. The Chi-squared test was used to compare the prevalence of hypertriglyceridemia in both diet groups. Pearson product-moment correlations were applied to correlate GLUT2, insulin, HOMA-IR, QUICKI, VLDL total mass, VLDL total lipids, PI3K, pAKT2^{ser473} and LDLr. Differences were considered significant at $p < 0.05$. Statistical analysis was performed using SPSS version 25.0 (SPSS Inc., Chicago, Illinois, USA).

3. Results

3.1. Growth rate, feed consumption and fecal excretion

Table 2 summarizes the feed intake, growth rate and fecal excretion of both groups. CE animals showed significant differences respect to C group in the growth rate, fecal excretion, fecal moisture, fecal fat and dietary digestibility ($p \leq 0.014$). CE rats displayed lower growth rate and dietary digestibility than C animals. Fecal excretion, fecal fat and fecal moisture were significantly higher in CE rats respect to C group (10.1%, 31.4% and 72.4%, respectively).

3.2. Plasma analysis

Table 3 shows glycemia, insulinemia, HOMA-IR, QUICKI, plasma lipid concentrations, atherogenic index (AI) and the cholesterol-to-phospholipid and cholesterol-to-HDL cholesterol ratios in the two experimental groups. C rats showed a typical T2DM status with fasting hyperglycemia, hyperinsulinemia and high levels of HOMA-IR. Insulin, HOMA-IR, QUICKI, triglycerides, total lipids and AI were significantly affected by CFE-RM diet ($p \leq 0.028$). CE rats revealed significantly lower insulin (−11.5%), HOMA-IR (−15.7%), triglycerides (−15.2%), total lipids (−10.8%) and AI (−34.4%) than C animals, while QUICKI

Table 2
Feed intake, growth rate and fecal excretion of animals fed different diets.

	C	CE	Student's *t*-test
Dietary intake (g/wk)	115.8 ± 8.52	101.0 ± 6.69	p = 0.002
Growth rate*	0.20 ± 0.01	0.18 ± 0.01	p < 0.001
Final weight (g)	388.3 ± 38.9	377.6 ± 32.2	p > 0.05
Fecal excretion (g/wk)**	7.99 ± 0.56	8.80 ± 0.29	p = 0.014
Fecal moisture (%)	14.35 ± 1.12	18.86 ± 1.21	p < 0.001
Fecal fat (mg/g faeces)**	80.00 ± 6.66	137.89 ± 7.15	p < 0.001
Dietary digestibility***	0.92 ± 0.01	0.90 ± 0.01	p = 0.001

Data are expressed as the mean ± standard deviation (n = 8).
* Growth rate: g food converted in g body weight.
** Data are dry matter weights.
*** Dietary digestibility = (feed intake − faeces)/feed intake. Dietary intake was measured daily and body weight, once a week; feces were collected the last week of the study. C, rats fed diet with control restructured meat; CE, rats fed diet with CFE-enriched meat.

Table 3
Glycemia, insulin, plasma lipids, cholesterol:phospholipid and cholesterol:HDL-cholesterol ratio and atherogenic index of animals fed different diets.

	C	CE	Student's *t*-test
Glycemia 3rd week (mmol/L)	6.00 ± 0.44	6.44 ± 0.47	p > 0.05
Glycemia 8th week (mmol/L)	13.92 ± 0.91	13.23 ± 0.71	p > 0.05
Insulin (μIU/mL)	15.84 ± 0.73	14.02 ± 1.04	p = 0.002
HOMA-IR*	9.79 ± 0.64	8.25 ± 0.63	p < 0.001
QUICKI**	0.278 ± 0.002	0.284 ± 0.003	p < 0.001
Total cholesterol (mmol/L)	2.16 ± 0.18	2.06 ± 0.18	p > 0.05
Phospholipids (mmol/L)	1.35 ± 0.32	1.23 ± 0.24	p > 0.05
Triglycerides (mmol/L)	1.78 ± 0.25	1.51 ± 0.13	p = 0.017
Total lipids (mg/dL)+	332 ± 35.3	296 ± 18.5	p = 0.028
Cholesterol:phospholipids (mol/mol)	1.57 ± 0.29	1.65 ± 0.29	p > 0.05
Cholesterol:HDL cholesterol (mol/mol)	1.43 ± 0.22	1.28 ± 0.14	p > 0.05
Atherogenic index++	0.32 ± 0.06	0.21 ± 0.09	p = 0.014

Data are expressed as the mean ± standard deviation (n = 8).
* HOMA-IR: (fasting insulin (μIU/mL) × fasting glucose (mmol/L)/22.5).
** QUICKI: 1/[log (fasting insulin (μIU/mL) + log (fasting glucose (mg/dL)]).
\+ Total lipids: cholesterol + triglycerides + phospholipids.
\++ Atherogenic index = (total cholesterol − HDL cholesterol)/HDL cholesterol. To transform mmol/L into mg/dL of glucose, cholesterol, TGs, and phospholipids, multiply data by 18.0, 38.68, 89.0, and 75.0, respectively. All parameters were evaluated at the end of the study (8th week) with the exception of glycemia (3rd week). C, rats fed diet with control restructured meat; CE, rats fed diet with CFE-enriched meat.

index was significantly higher in CE *versus* C group ($p < 0.001$). Hypertriglyceridemia (≥1.7 mmol/L) was found in 62.5% of C rats, but 0% of CE rats (chi-square test, $p < 0.001$), showing a marked reduction in CE groups *versus* their C counterparts (−15.2%).

3.3. Lipoprotein composition

The composition of the different lipoprotein fractions in absolute values (mmol/L or mg/dL) is shown in Table 4. HDLs were the major carriers of cholesterol (75.7 *versus* 77.1%), phospholipids (72.7 *versus* 73.04%) and apolipoproteins (91.0 *versus* 85.7%) in C *versus* CE rats; whereas VLDLs were the major carrier of TGs (86.2 *versus* 70.8% in C and CE, respectively).

CE rats showed significantly ($p < 0.05$) lower content of cholesterol, triglycerides, phospholipids, total lipids and total mass in VLDL fraction with respect to C group. CE animals displayed higher triglycerides, phospholipids, total lipids, proteins and total mass in IDL and LDL lipoprotein fractions ($p = 0.015$) than their C counterparts. HDL lipoprotein fraction only exhibited differences in phospholipids, showing lower levels in CE rats respect to C ($p = 0.014$).

CE animals exhibited significantly lower total lipids/proteins and cholesterol/proteins ratios in VLDL and LDL fractions ($p = 0.001$); and lower total lipids/proteins of IDL than C rats. Non-significant differences ($p > 0.05$) were observed between C and CE groups in the IDL cholesterol/protein ratio and HDL of lipids and cholesterol ratios (data not-shown).

3.4. Percentage contribution of lipids to lipoprotein composition and liver LDLr levels and immunolocalization

Fig. 1(a)–(d) displays the percentage contribution of lipids and proteins to the total mass of plasma VLDL, IDL, LDL, and HDL. CE rats had less triglycerides (−3.4%) and more proteins (55.4%) in VLDL fraction than C group ($p = 0.041$). Cholesterol (99.0%) and proteins (169.6%) contributed more while phospholipids less (−40.1%) to the IDL total mass in CE *versus* C rats ($p = 0.002$). CE animals showed LDL

Table 4
Lipoprotein component concentrations in plasma of animals fed different diets.

Lipoproteins	C	CE	Student's *t*-test
Cholesterol, mmol/L			
VLDL	0.44 ± 0.05	0.35 ± 0.09	p = 0.005
IDL	0.02 ± 0.00	0.10 ± 0.02	p < 0.001
LDL	0.03 ± 0.00	0.03 ± 0.01	p > 0.05
HDL	1.53 ± 0.14	1.62 ± 0.14	p > 0.05
Triglycerides, mmol/L			
VLDL	1.56 ± 0.05	0.97 ± 0.06	p < 0.001
IDL	0.08 ± 0.04	0.19 ± 0.05	p < 0.001
LDL	0.03 ± 0.01	0.05 ± 0.01	p = 0.014
HDL	0.14 ± 0.01	0.16 ± 0.02	p > 0.05
Phospholipids, mmol/L			
VLDL	0.31 ± 0.03	0.20 ± 0.04	p < 0.001
IDL	0.05 ± 0.01	0.09 ± 0.01	p < 0.001
LDL	0.02 ± 0.00	0.09 ± 0.01	p < 0.001
HDL	1.01 ± 0.15	0.84 ± 0.11	p = 0.014
*Total lipids, mg/dL**			
VLDL	178.60 ± 6.12	113.22 ± 10.20	p < 0.001
IDL	11.56 ± 3.69	27.50 ± 4.87	p < 0.001
LDL	4.93 ± 1.43	6.63 ± 0.96	p = 0.015
HDL	145 ± 12.10	137 ± 11.58	p > 0.05
Proteins, mg/dL			
VLDL	5.07 ± 0.81	5.05 ± 0.56	p > 0.05
IDL	0.57 ± 0.26	3.88 ± 0.87	p < 0.001
LDL	0.40 ± 0.14	1.08 ± 0.45	p = 0.003
HDL	60.9 ± 5.43	60.1 ± 3.12	p > 0.05
*Total lipoprotein mass, mg/dL***			
VLDL	184 ± 6.44	118 ± 10.50	p < 0.001
IDL	12.05 ± 3.91	30.83 ± 5.78	p < 0.001
LDL	5.32 ± 1.49	7.72 ± 1.08	p = 0.003
HDL	206 ± 15.96	197 ± 14.14	p > 0.05

Data are expressed as the mean ± standard deviation (n = 8).
* Total lipids: cholesterol + triglycerides + phospholipids.
** Total mass: total lipids + proteins. All samples were taken at the end of 8th week experiment. C, rats fed diet with control restructured meat; CE, rats fed diet with CFE-enriched meat.

with less cholesterol (−38.1%) and phospholipids (−24.8%) but more proteins (82.7%) than C counterparts ($p = 0.010$). HDL total mass revealed more cholesterol (10.5%) and less phospholipids (−12.9%) contributions in CE *versus* C rats ($p = 0.003$).

The CFE-enriched meat effects on liver LDLr levels and immunolocalization are shown in Figs. 2 and 3(a) and (b). CE rats revealed higher levels (12.9%) and immunohistochemical staining (37.8%) than C animals ($p < 0.05$). Immunoblots from CE rats displayed higher LDLr levels (12.9%) compared to C animals ($p < 0.05$). Immunohistochemistry revealed a diffuse and very faint cytoplasmic staining pattern in sections from C liver using a monoclonal anti-LDLr antibody. Punctate most intense and stronger LDLr-specific staining was seen in the CE liver sections, showing a panlobular pattern. CE induced a higher LDLr immunoreactivity (37.8%) compared with C liver sections.

3.5. Arylesterase activity, VLDL and liver oxidation

Table 5 shows the plasma AE activity, the AE activity-to-plasma cholesterol ratio, liver AE activity and VLDL and liver oxidations. Plasma AE activity and AE activity-to-plasma cholesterol ratio were significantly reduced by CE diet (−19.5 and −16.7%, respectively; $p = 0.024$). Instead, a significant increase was observed in the liver AE activity (23.6%; $p = 0.037$) in CE compared to C rats. Non-significant differences were observed ($p > 0.05$) in VLDL-ox and liver-ox between groups.

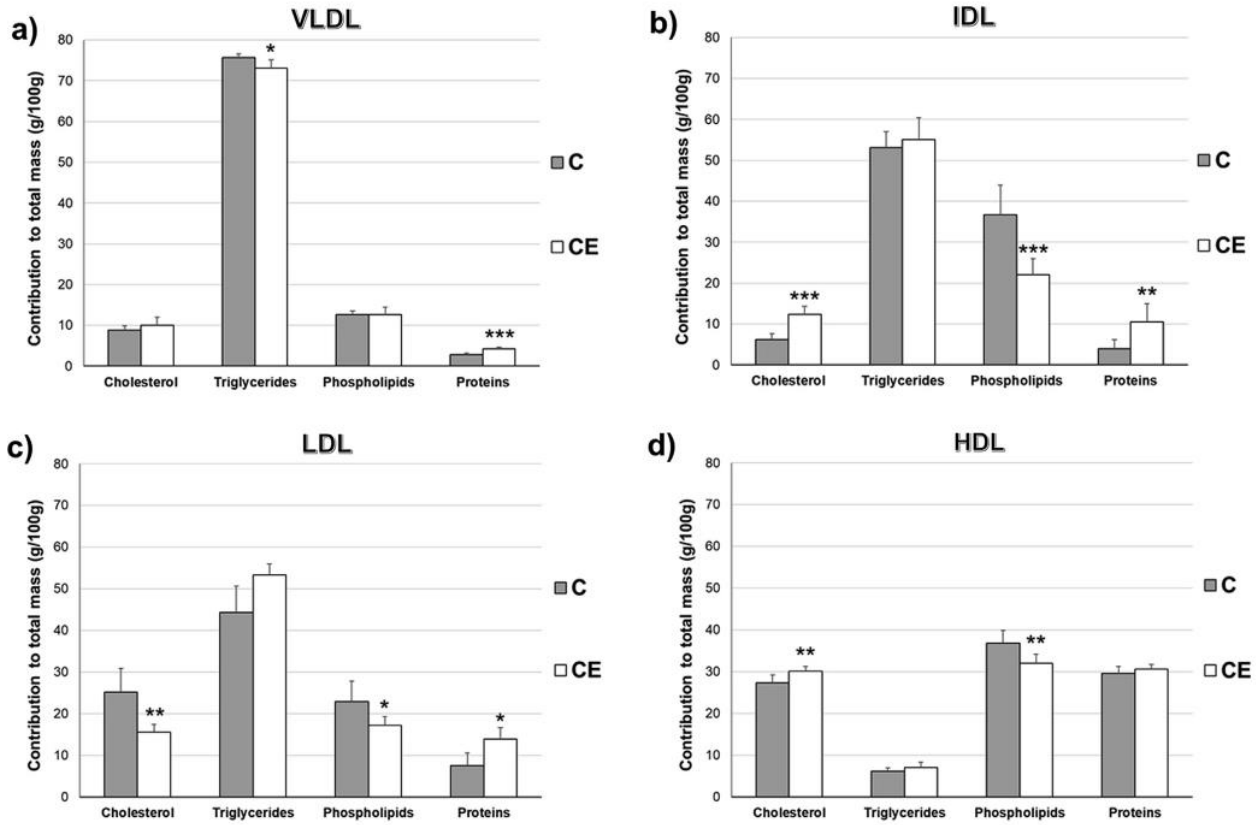

Fig. 1. Percentage contribution (%) of proteins and lipids to the total mass of plasma VLDL (a), IDL (b), LDL (c) and HDL (d) fractions in the C and CE groups. Data are expressed as the mean ± standard deviation (n = 8). *p < 0.05; **p < 0.01; ***p < 0.001. C, rats fed diet with control restructured meat; CE, rats fed diet with CFE-enriched meat.

3.6. Insulin signaling (InsR/PI3K/AKT/GSK3 pathway) analyzed by western blot and immunohistochemistry

Fig. 3(a)–(d) displays the levels and distribution of the main proteins related to liver insulin signaling pathway (InsR/PI3K/AKT/GSK3 pathway). CE rats showed higher protein expression levels of PI3K (WB = 29.3%), pAKT2^{ser473} (WB = 13.1%) and GLUT2 (WB = 33.9%) than C animals. The immunohistochemistry analysis data supported the quantitative western blot results. A homogeneously distributed staining for InsR, PI3K, pAKT2^{ser473} and pGSK3β^{ser9}, which was slightly stronger

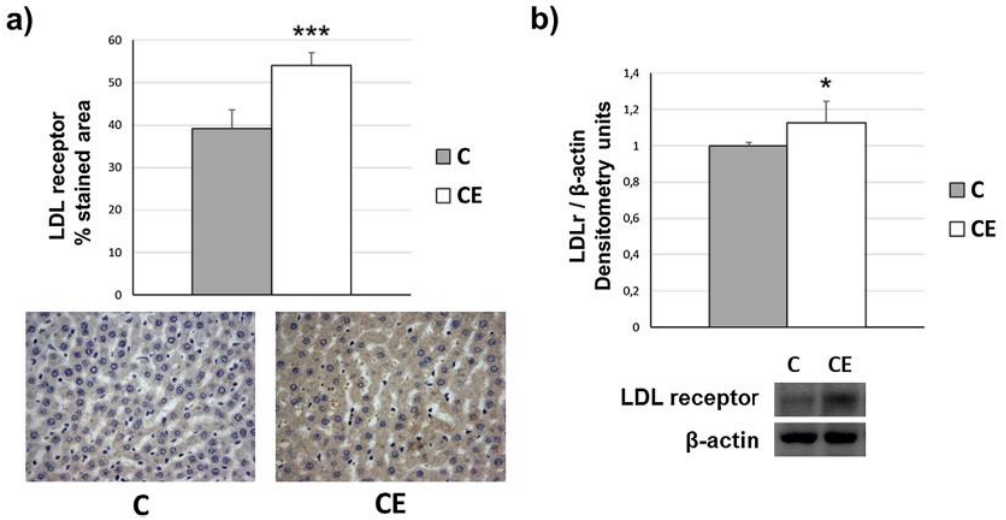

Fig. 2. Liver LDL-receptor levels and localization in the different groups. Data are expressed as the mean ± standard deviation (n = 8). (a) LDLr immunohistochemical staining quantification and representative images of the different groups; (b) LDLr levels quantification by western blot. *p < 0.05; **p < 0.01; ***p < 0.001. C, rats fed diet with control restructured meat; CE, rats fed diet with CFE-enriched meat.

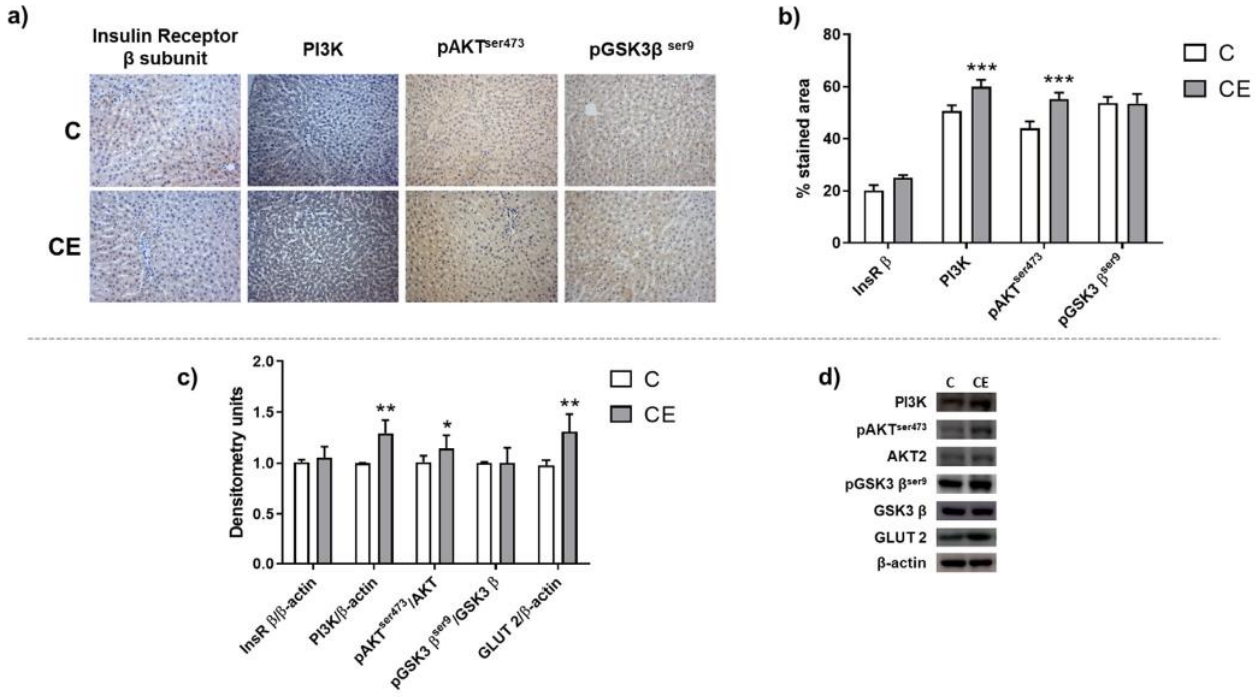

Fig. 3. Levels and localization of the main proteins related to InsR/PI3K/AKT/GSK3 pathway of animals fed the experimental diets. Data are expressed as the mean ± standard deviation (n = 8). (a) representative images of liver immunohistochemical staining for the InsR/PI3K/AKT/GSK3 pathway; (b) staining area quantification for each of the analyzed proteins; (c) quantification of InsR/PI3K/AKT/GSK3 levels in liver extracts by western blot; (d) representative images of the different western blot realized. *p < 0.05; **p < 0.01; ***p < 0.001. C, rats fed diet with control restructured meat; CE, rats fed diet with CFE-enriched meat.

Table 5
LDL receptor levels, plasma and liver AE activity and VLDL and liver oxidation of animals fed the experimental diets.

	C	CE	Student's *t*-test
Plasma AE (U/L)	185 ± 38.9	149 ± 15.8	p = 0.006
Plasma AE:cholesterol (U/mg)*	0.24 ± 0.06	0.20 ± 0.02	p = 0.024
Liver AE (U/g protein)	6.11 ± 1.05	7.55 ± 1.24	p = 0.037
VLDL-ox (TBARS, mg MDA/L)	2.18 ± 0.24	2.23 ± 0.22	p > 0.05
Liver-ox (TBARS, mg MDA/mg protein)	2.62 ± 0.44	2.52 ± 0.26	p > 0.05

Data are expressed as the mean ± standard deviation (n = 8).

* Units AE (U/L)/total cholesterol (mg/L). One unit of AE was defined as mmols of phenol formed from phenylacetate per minute. All samples were taken at the end of 8th week experiment. C, rats fed diet with control restructured meat; CE, rats fed diet with CFE-enriched meat.

in centrilobular areas, was observed in liver sections from both groups. The CE livers showed a stronger PI3K (18.4%) and pAKT2^{ser473} (25.3%) staining and bigger surface area in hepatocytes around periportal and pericentral areas, suggesting PI3K and pAKT2^{ser473} over-expression. Non-significant differences between C and CE groups were found for InsR and pGSK3β^{ser9} proteins.

4. Discussion

These results shed new light on the role of CFE and its utilization to develop functional meats to reduce qualitative, quantitative and kinetic lipoprotein alterations by improving insulin signaling in T2DM rats fed a high-saturated-fat diet. CFE-enriched meat partially blocked T2DM alterations, reduced insulinemina, HOMA-IR, triglyceridemia and VLDL levels and composition; and increased fecal fat excretion, LDLr levels and insulin signaling in the liver (Fig. 4). These results are relevant given the high consumption of meat in Western countries and the low fiber and polyphenols intakes observed in diabetic population. Therefore, it can be suggested the possibility of reducing T2DM alterations and increasing fiber intake, by including the CFE as a functional ingredient in meat products.

Experimental diets were well accepted and agreed with previous results of our group on functional meat products (Macho-González et al., 2019; Santos-López et al., 2018). Although the final weight of rats was very similar in both groups, the lower dietary intake and dietary digestibility of CE group justify the lower growth rate of those rats. These results seem primarily due to a satiating effect and increase of fecal bolus mediated by fiber (Macho-González et al., 2018; McRae, 2018). Likewise, differences in fecal amount and composition should be related to CFE proanthocyanidins. Similar results were previously found in a digestibility study in healthy and diabetic rats (Macho-González et al., 2018, 2019). Proanthocyanidins have also been related to gastric emptying delay. Together with the fiber satiating effect, these two components would increase satiety and therefore a lower diet intake, as observed in CE animals (Cires, Wong, Carrasco-Pozo, & Gotteland, 2016; Serrano et al., 2016).

Feeding with a HSF diet induces IR in different animal models (Sah et al., 2016; Skovsø, 2014). As observed in our results, the C rats present hyperglycemia, hyperinsulinemia, and typical dyslipemia at early T2DM stage (Asrafuzzaman et al., 2017; Fang et al., 2019). CE animals displayed lower HOMA-IR and higher QUICKI mainly due to their insulinemia being significantly lower than that of C group and linked to the proanthocyanidins effect on carbohydrates metabolism, as reported by Yang et al. (Yang & Chan, 2017). Furthermore, proanthocyanidin-consumption, by diminishing both glucose digestion and absorption, improves IR by reducing glycemia and insulinemia (Cires et al., 2016; Salvado, Casanova, Fernandez-Iglesias, Arola, & Blade, 2015; Yang & Chan, 2017). In fact, a lower glycemia increase (−15%) was observed between the third and eighth week in CE rats (6.79 *versus* 7.92 mmol/L), which was consistent with the hypoglycemic effect observed in streptozotocin-nicotinamide diabetic rats (Macho-González et al., 2019)

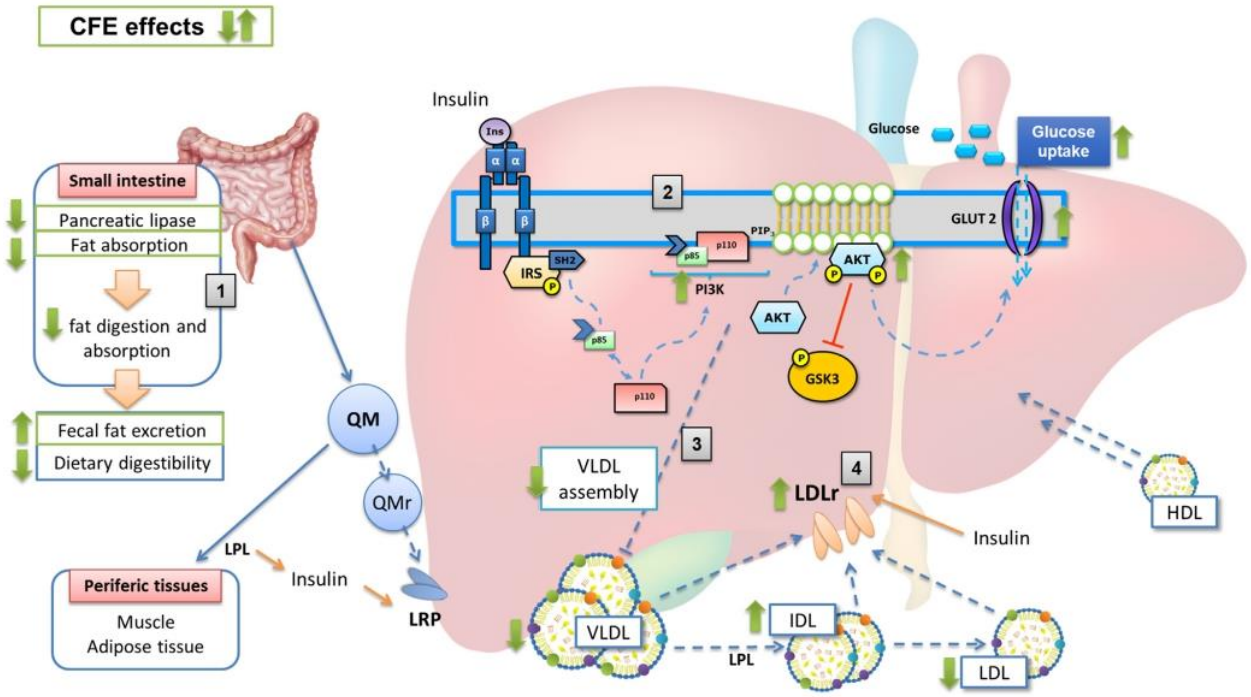

Fig. 4. Proposed mechanism of the carob fruit extract (CFE) effects on lipoprotein metabolism and insulin signaling. The T2DM alterations induced by high-saturated-fat diet consumption are based on hyperglycemia, hyperinsulinemia and insulin resistance. (1) The CFE-enriched meat consumption increases fat excretion by decreasing digestion and absorption of lipids; (2) the main proteins levels involved in insulin signaling increased in the group fed with CFE, which would improve glucose uptake; (3) the lower plasma triglycerides availability to form VLDL, together with the InsR/PI3K/AKT pathway inhibition on the VLDL assembly and maturation, justify the lower levels and size of these lipoproteins; (4) the CFE increases LDLr levels, promoting the uptake of IDL and LDL. AKT; CFE, carob fruit extract; HDL, high-density lipoproteins; GLUT2, glucose transporter 2; GSK3, glycogen synthase kinase 3; IDL, intermediate-density lipoproteins; Ins, insulin receptor; IRS, insulin receptor substrate; LDL, low-density lipoproteins; LDLr, low density lipoprotein receptor; LPL, lipoprotein lipase; LRP, low density lipoprotein receptor-related protein; PI3K, phosphoinositide 3-kinase (formed by p85 and p110 subunits); QM, chylomicrons; QMr, remnant chylomicrons; VLDL, very low-density lipoproteins.

and in chow fed rats during a postprandial study (Macho-González et al., 2017).

Diabetic dyslipidemia is the main and more frequently lipid abnormality observed in T2DM patients (Verges, 2015). Hypertriglyceridemia is considered one of the basic T2DM dyslipidemia pillars, being the promoter of quantitative and qualitative lipoprotein changes (Verges, 2015). Using 1.7 mmol/L as a cut-off point of hypertriglyceridemia (Berglund et al., 2012), 62.5% of the C rats while 0% of CE animals presented hypertriglyceridemia. These results agree with previous studies of our group in healthy rats, where CFE-consumption reduced postprandial hypertriglyceridemia (Macho-González et al., 2018), and with studies carried out in humans (Goulas, Stylos, Chatziathanasiadou, Mavromoustakos, & Tzakos, 2016).

The lower plasma triglyceride, total lipid and AI observed in CE rats seem to be due to the conjoint action of proanthocyanidins (reducing fat digestion and absorption), and the drag effect of fiber (Kaline et al., 2007; Salvado et al., 2015). Thus, the inclusion of CFE-enriched meat in the diet seems to be an adequate nutritional strategy to ensure the fiber and polyphenols intakes, usually reduced in T2DM patients (McRae, 2018). Together with the hypertriglyceridemia, C rats showed higher total lipids and AI than CE rats. Results of C group on lipoprotein fractions indicate VLDL qualitative and quantitative changes in comparison with previous studies in healthy rats (Schultz-Moreira et al., 2014). C animals showed the typical lipoprotein profile of diabetic rats with the presence of $VLDL_1$, characterized by a larger size and by being triglycerides-enriched (Haas, Attie, & Biddinger, 2013; Verges, 2015). In fact, isolated VLDL in C rats contain 4.2 and 3.2 times more triglycerides and their total lipid masses were 3.8 and 2.6 times higher than those found by Schultz-Moreira et al. and Viejo, respectively (Schultz-Moreira et al., 2014; Viejo, 1992), suggesting a VLDL overproduction or lower VLDL catabolism. On the other hand, CE rats display 36% less total VLDL mass than C rats. In addition, these VLDL particles seem to be smaller and in a more advanced catabolism, because of their lower content in triglycerides while higher in proteins (Fig. 1). Related to this, an increase in total IDL and LDL mass was clearly observed in CE group. A detailed study of IDL composition suggests that they were enriched in cholesterol and impoverished in phospholipids. These results may be due to lipoprotein kinetic modifications, proper of the diabetic situation in which a decrease in lipoprotein remnant catabolism has been suggested (Parhofer, 2011; Verges, 2015). In order to reduce this remnant lipoprotein amount, higher LDLr levels were found in CE rats, a fact that has also been observed in a previous study with diabetic and hypercholesterolemic rats (Macho-González et al., 2019). Finally, diabetic dyslipidemia is also related to quantitative and qualitative changes in the HDL fraction (Tomkin & Owens, 2017). However, in our study we barely observed relevant modifications in the HDL fraction, just a relative cholesterol-enrichment and phospholipid-impoverished of HDL particles in CE group with respect to C, which could be associated with greater cholesterol elimination from the tissues (Barter, 2002).

The PON-1 has been defined as a suicide enzyme with pleiotropic antioxidant action (Aviram, 2004). According to Olivero-David et al. (Olivero-David et al., 2011) this enzyme acts first to protect the presence of other antioxidant enzymes. As CFE shows antioxidant activity (Bastida et al., 2009) it can be speculated that less AE is demanded to protect the antioxidant status in CE rats *versus* C rats. Estrada-Luna et al. (Estrada-Luna et al., 2018) found that rats fed antioxidants show a decrease in the AE activity. The higher liver AE activity of CE animals seems related to the enhanced lipoprotein catabolism of this group, in

order to reduce the radical oxygen species formation during fatty acids oxidation. However, more research has to be performed to ascertain this potential relationship.

In order to find the relationship between diabetic dyslipidemia and insulinemia, we evaluated hepatic insulin-signaling as the central axis of the altered lipoprotein metabolism (Haas et al., 2013; Verges, 2015). Several studies indicate that the Ins/PI3K/AKT pathway modulates the insulin effect on lipoproteins metabolism, and its alteration may produce a VLDL overproduction (Brown & Gibbons, 2001; Taghibiglou et al., 2002) and hepatic LDLr reduction (Duvillard et al., 2003), results that explain, at least partially, C rat data. CE rats presented higher PI3K and $pAKT2^{ser473}$ levels than their C counterparts, suggesting that a CE diet promotes a more effective insulin signals transduction than the C diet. The insulin binding to its receptor causes the recruitment of proteins from the insulin receptor substrate and the subsequent PI3K translocation, inducing many of the known metabolic insulin effects. PI3K activates AKT, which phosphorylates and inhibits GSK3, favoring glucose uptake and glycogen synthesis (Lee & Kim, 2007). This signaling pathway is affected by IR and the proanthocyanidins consumption may improve its activation in order to manage glucose homeostasis (Salvado et al., 2015). The Ins/PI3K/AKT pathway participates in the assembly and VLDL maturation, by inhibiting the lipids transfer to the VLDL precursors (Sparks, Sparks, & Adeli, 2012) and justifies the lower VLDL amount and size in CE rats. Likewise, the improvement in insulin signaling induces the LDLr expression, as observed and previously discussed in rats fed CFE-enriched meat (Macho-González et al., 2019). On the other hand, the PI3K and $pAKT2^{ser473}$ higher levels in CE rat liver are also related to better glucose uptake. Proanthocyanidin-consumption has shown beneficial effects in glycemia by improving glucose uptake in different tissues through the AKT2 phosphorylation (Salvado et al., 2015; Yang & Chan, 2017). Proanthocyanidin consumption has been associated with an increase in liver GLUT2 levels (Cao et al., 2016). In the present study CE rats showed a 31% higher GLUT2 levels *versus* those C animals, being a clear indication of improved glucose uptake and insulin-sensitivity. In fact, a strong negative and significant correlation ($r = -0.696$; $p < 0.01$) was found between GLUT2 and HOMA-IR. A detailed study of data suggests a very high relationship between insulin-signaling, glucose homeostasis and VLDL levels. Thus, PI3K and $pAKT2^{ser473}$ quantified by immunohistochemistry were negatively and very significantly correlated ($r = -0.700$; $p < 0.01$) with VLDL mass, VLDL lipids, insulin and HOMA-IR; and positively and significantly correlated with LDLr, GLUT2 and QUICKI ($r = 0.648$; $p < 0.05$).

Despite the positive results observed, this study presents some possible limitations: (1) only the study has been carried out in males, (2) healthy control could be added to the study, and (3) the study lasted only 8 weeks. As T2DM is a chronic degenerative disease, future researches should analyze the CFE-enriched meat effect in animal model longitudinal studies. In addition, as CFE has been found to be safe in healthy and diabetic rats (Macho-González et al., 2017, 2018) and in hypercholesterolemic human (Ruiz-Roso, Quintela, de la Fuente, Haya, & Perez-Olleros, 2010), it seems desirable to conduct a clinical trial in prediabetic and/or T2DM patients.

In conclusion, present results show for the first time that a diet containing meat formulated with CFE improves both lipoprotein metabolism and InsR/PI3K/AKT pathway as a consequence of insulin sensitivity-ameliorating. Fundamentally, CFE reduces VLDL and plasma triglyceride levels by increasing fecal fat excretion, and permitting to increase the effectiveness of the insulin signaling. At the same time, it favors the clearance of atherogenic lipoproteins, by increasing hepatic LDLr (Macho-González et al., 2019). Therefore, we can suggest that CFE-enriched meat is adequate functional food to be consumed by T2DM patients to control diabetic dyslipidemia and IR.

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Declaration of Competing Interest

No conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103600.

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Publicación 8

Can Carob-Fruit-Extract-Enriched Meat Improve the Lipoprotein Profile, VLDL-Oxidation, and LDL Receptor Levels Induced by an Atherogenic Diet in STZ-NAD-Diabetic Rats?

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Antecedentes: La dislipemia diabética es otra de las alteraciones que normalmente sufren los pacientes con DMT2. Se caracteriza por anormalidades cuantitativas, cualitativas y cinéticas en el metabolismo de las lipoproteínas, entre las que se destaca un incremento de las VLDL y un menor aclaramiento de partículas LDL, con la consiguiente formación de sdLDL susceptibles de oxidación (Parhofer, 2011; Verges, 2015). El CFE ha demostrado propiedades hipolipemiantes en sujetos hipercolesterolémicos (Ruiz-Roso y cols., 2010), resultados que fueron corroborados en los estudios descritos en el capítulo 1 en ratas Wistar sanas. A pesar de estos buenos resultados, el efecto del CFE sobre la alteración en el metabolismo de las lipoproteínas de sujetos con DMT2 nunca ha sido probada. Por ello, evaluar el efecto del consumo de un cárnico con CFE sobre las modificaciones cualitativas y cuantitativas de las lipoproteínas en un modelo de DMT2 es altamente relevante.

Hipótesis: La adición de CFE como ingrediente funcional incluido en una matriz cárnica minimiza las alteraciones en el perfil lipoproteico provocadas por el consumo de una dieta alta en grasas saturadas y colesterol en un modelo de DMT2 avanzada.

Resultados: Los tres grupos de estudio (ED, D y DE) mostraron niveles equivalentes de ingesta y peso corporal, lo que demuestra la adecuada aceptación de las dietas. Sin embargo, los animales ED y DE revelaron una menor digestibilidad de la dieta, debido fundamentalmente a la mayor excreción fecal y presencia de grasa en heces encontrada en estos grupos. Ambos grupos que consumieron el cárnico enriquecido en CFE, mostraron una reducción significativa de la glucemia y la colesterolemia en comparación con el grupo control. El grupo D mostró el perfil típico de lipoproteínas de ratas hipercolesterolémicas, mientras que los animales que recibieron CFE bloquearon parcialmente este efecto, mostrando niveles más bajos de β-VLDL y LDL, así como presencia de HDL típicas, con mayor contenido de colesterol. Por otra parte, se encontrarón niveles incrementados del LDLr hepático en los animales ED y DE. Por último, la actividad AE fue significativamente superior en los grupos alimentados con CFE, lo que se correspondió con niveles reducidos de VLDL-ox y oxidación hepática en comparación con los observados en el grupo D.

Conclusiones: El consumo de un cárnico enriquecido en CFE reduce los efectos negativos de una dieta aterogénica al mejorar la glucemia, lipemia, actividad arilesterasa, niveles LDLr y el perfil lipoproteico, especialmente cuando se consume como estrategia estrategia preventiva. Por tanto, la inclusión de CFE como ingrediente funcional en un reestructurado cárnico parece ser una adecuada estrategia nutricional para reducir la dislipemia diabética en pacientes con prediabetes y DMT2.

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Article

Can Carob-Fruit-Extract-Enriched Meat Improve the Lipoprotein Profile, VLDL-Oxidation, and LDL Receptor Levels Induced by an Atherogenic Diet in STZ-NAD-Diabetic Rats?

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Abstract: Carob fruit extract (CFE) has shown remarkable in vitro antioxidant properties and reduces postprandial hyperglycemia and hyperlipidemia in healthy animals. Development of functional meat products that contain bioactive components are presented as a great nutritional strategy. Until now, the effect of the consumption of restructured meat enriched with CFE in a murine model of diabetes has not been investigated. The objective of this study was to evaluate the effect on glycemia, lipemia, lipoprotein profile, *Ldlr*, arylesterase (AE), and very low-density lipoproteins (VLDL) and liver oxidation in streptozotocin-nicotinamide (STZ-NAD) growing Wistar diabetic rats fed restructured meat in the frame of a high cholesterol/high saturated-fat diet. In the present study, three groups (D, ED and DE) were fed cholesterol-enriched (1.4% cholesterol and 0.2% cholic acid) and high saturated-fat diets (50% of total energy from fats and 20.4% from saturated fatty acids). Rats were subjected to a STZ-NAD administration at the 3rd week. Group D did not receive CFE, while ED and DE rat groups received CFE before and after the diabetic induction, respectively. After eight weeks, D rats showed hyperglycemia and hypercholesterolemia, an increased amount cholesterol-enriched VLDL (β-VLDL), IDL and LDL particles and triglyceride-enriched HDL. ED and DE partially blocked the hypercholesterolemic induction with respect to D group ($p < 0.001$) and improved glycemia, cholesterol levels, lipoprotein profile, *Ldlr*, plasma AE activity and liver oxidation ($p < 0.001$). Fecal fat, moisture and excretion were higher while dietary digestibility was lower in ED and DE vs. D counterparts ($p < 0.001$). In conclusion, CFE-enriched meat shows, for the first time, hypoglycemic and hypolipidemic effects in STZ-NAD animals fed high cholesterol/high saturated-fat diets. Likewise, it manages to reverse possible diabetes lipoprotein alterations if CFE-enriched meat is consumed before pathology development or improves said modifications if Type 2 Diabetes Mellitus is already established.

Keywords: STZ-NAD; diabetes; carob fruit; functional meat; lipoproteins; LDL-receptor; lipoprotein oxidation

1. Introduction

Meat products are widely consumed in developed countries because they are a great source of protein, vitamins, and minerals [1]. However, it has been observed that a high intake of processed meat products or red meat greatly increases type 2 diabetes mellitus (T2DM) risk [2,3]. Although T2DM patients partially lose their ability to secrete insulin in response to carbohydrates due to insulin-resistance, they improve this hormone secretion when a meal contains high protein and amino acid levels [4,5].

On the other hand, T2DM patients usually suffer from dyslipemia, which includes quantitative, qualitative and kinetic lipoprotein abnormalities that, together, result in a shift towards a more atherogenic lipid profile [6,7]. These alterations are generally characterized in man by an increase in the level of very low-density lipoproteins (VLDL), small and dense low-density lipoproteins (LDL), as well as a high content of triglycerides in LDL and high-density lipoproteins (HDL), and an increased susceptibility of LDL to oxidation [6,8]. In previous studies, we observed that dietary cholesterol greatly increased the amount of plasma cholesterol in rodents giving rise to an increase of VLDL and LDL-cholesterol-enriched particles and to a decrease of HDL-cholesterol [9–13]. However, the effect of a high-cholesterol/high fat/high saturated fat diet in a T2DM model has been scarcely tested.

A key factor for the treatment of this pathology is dietetic intervention, and numerous studies have linked functional food consumption with an improvement of T2DM [14,15]. Thus, some functional foods, through the bioactive compounds they contain, could affect carbohydrate metabolism, giving rise to less marked postprandial hyperglycemia, improved pancreatic β-cell function and insulin secretion, as well as decreased insulin resistance [16]. Moreover, they could regulate lipid and lipoprotein metabolism and adipose tissue metabolism, modulate oxidative/antioxidative balance and inflammatory processes, improve weight management and prevent micro- and macro-vascular complications [17–19].

Our group has been working for years in functional foods and has extensive experience in the qualitative/quantitative modification of meat matrices with different active compounds, aimed at decreasing the potential negative effects on cardiovascular disease (CVD) risk factors of a high meat product consumption [9–13]. Thus, we have confirmed that the consumption of restructured meat enriched with walnut paste improves the antioxidant and lipoprotein profiles of at-risk CVD volunteers [20]. Similar data were also observed in Wistar rats fed restructured pork with *Himanthalia elongata* added [10]. Likewise, restructured pork enriched with silicon was able to partially block dyslipidemia induced by an obesogenic diet and reduce VLDL oxidation and improve liver arylesterase (AE) activity [9]. Now we have focused our attention on the development of a meat product enriched with carob fruit extract (CFE) because we have proven that it has hypoglycemic and hypolipemic properties in healthy animals [21,22]. Likewise, although CFE has been found to reduce oxidative alterations during meat storage [23], and to work as an antioxidant at frying temperatures [24], as far as we know, CFE has never been tested as a potential functional ingredient for meat products addressed at improving lipoprotein composition in streptozotocin plus nicotinamide (STZ-NAD) rats as an animal model of diabetes [25,26].

CFE is a purified fraction of carob pulp of the *Ceratonia siliqua L.* variety. Its composition is mainly based on 71–81% insoluble fiber [27]. CFE has a large amount of insoluble polyphenols and specifically high molecular-weight proanthocyanidins to which numerous beneficial health effects have been attributed [28]. Therefore, this study hypothesized that the addition of CFE as a functional ingredient to a meat matrix attenuates lipoprotein alteration induced by a high cholesterol/high saturated-fat diet in diabetic Wistar rats. In addition, we hypothesized that the CFE effects will be better when this functional ingredient is consumed a few weeks before STZ-NAD diabetic induction than they would be just after induction is confirmed. Therefore, the main objectives were to assess, in these rat models: (1) growth rate, (2) lipid and lipoprotein profiles, (3) VLDL oxidation, (4) plasma and liver arylesterase (AE) activities, (5) liver LDL receptor (*Ldlr*) by Western blot and immunohistochemistry analysis, and (6) to compare CFE effects before and after STZ-NAD diabetic induction.

2. Materials and Methods

2.1. Carob Fruit Extract (CFE)

CFE is a natural insoluble dietary fiber, which is obtained from carob pulp following the procedure described in patent WO2004/014150 [27] and Macho-González et al. [22]. Briefly, its major compound is total dietary fiber, 74–84%, of which the soluble fraction accounts for 1–3% and the insoluble one for 71–81%. CFE has a polyphenols concentration of non-extractable condensed tannins of 34–40% and soluble extractable polyphenols of 0.5–1%, according to patent WO2006/000551 [29].

2.2. Restructured Meat and Diet Preparation

Diets were prepared from a purified diet formulation (reference U8959, version 180; Panlab S.L.). Briefly, mixed minced meat (50% pork:50% veal) and lard were purchased at a local store. Restructured meat (RM) was prepared following the protocol described by Schultz-Moreira et al. [30]. For RM with CFE (CFE-RM), CFE (4 g/kg restructured meat) was homogenized with lean mixed meat. The resulting RMs were freeze-dried and ground in a chilled meat cutter (Stephan Universal Machine UM5; Stephan, Shóne Gmbh and Co.) following a standard procedure to obtain a powder product (Supplementary Table S1) [30]. For each kilogram of diet, 30% of RM, 5% of cellulose powder and 65% of a purified diet formulation were mixed and subsequently sieved 3 times until a completely homogenous powder was obtained. Diets were designed to contain the following: Control: 50 % energy from fat (20.4 % saturated fat), 36 % energy from carbohydrate, and 14 % energy from protein. Two experimental semisynthetic diets were prepared: (a) Diet containing the control-RM and 1.4% cholesterol and 0.2% cholic acid (98% purity) (Chol diet); and (b) identical to the Chol diet but containing CFE-RM (CFE diet). Diets designed with CFE-RM contained 1.14% less dietary cellulose with the objective of keeping similar total amount of fiber in diets (Table 1).

Table 1. Composition of the experimental diets fed to Wistar rats.

Dietary Components	Chol-Diet	CFE-Diet
Protein, %	17.03	17.03
Fat, %	27.02	27.02
Cholesterol, g/kg	9.83	9.83
SFA:MUFA:PUFA ratio	2.76/2.99/1	2.76/2.99/1
Energy content *, MJ/kg	20.36	20.36
Ingredients, g/kg		
Sucrose	68.25	68.25
Corn starch	275.73	275.73
Casein	94.25	94.25
Maltodextrin	94.25	94.25
Cellulose	48.86	48.86
PM 205B SAFE	50.05	50.05
PV 200 SAFE	7.15	7.15
Soybean oil	47.91	47.91
L-Cysteine	2.02	2.02
Cholesterol	9.1	9.1
Cholic acid	1.3	1.3
Freeze-dried restructured meat	301.14	301.14

* Data were calculated according to the energy equivalents for carbohydrate (16.73 kJ/g (4.0 kcal/g)), fat (37.65 kJ/g (9.0 kcal/g)), and protein (16.73 kJ/g (4.0 kcal/g)). Chol-diet, diet containing the control-RM and 1.4% cholesterol and 0.2% cholic acid; CFE-diet, diet containing the carob fruit extract-RM (CFE-RM) and 1.4% cholesterol and 0.2% cholic acid. SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; PM 205B SAFE, Mineral mix; PV 200 SAFE, Vitamin Mix.

2.3. Experimental Design

Experiments were performed in compliance with directive 86/609/EEC of 24 November, 1986 (amended by Directive 2003/65/EEC of 22 July, 2003), on the protection of scientific research animals. The study was approved by the Spanish Science and Technology Advisory Committee (project AGL2014-53207-C2-2-R) and by the Ethics Committee of the Universidad Complutense de Madrid (Spain).

Twenty-four male Wistar rats aged two-months were obtained from Harlan S.L. (Barcelona, Spain) and housed in couples under a controlled temperature (22.3 ± 1.9 °C) and light (12-h light/dark cycle) at the Centro de Experimentación Animal of the University of Alcalá, Madrid, Spain (register no. ES280050001165). Food and tap water were provided ad libitum. Rats were divided into 3 groups of 8 animals each. At the 3rd week of the study T2DM was induced by intraperitoneal injection of streptozotocin (STZ, 65 mg/kg body weight, b.w.) and nicotinamide (NAD, 225 mg/kg b.w.) (both from Sigma-Aldrich, Madrid, Spain) [25,26]. Briefly, animals were divided into 3 groups: (1) Rats fed the Chol diet (D); (2) rats fed the CFE diet from the beginning of the study (ED); (3) rats fed the CFE diet when the diabetic state was confirmed (DE). The study lasted 8 weeks.

In order to avoid inter-assay variations, overnight fasted rats were taken one at a time from each of the three groups at the end of experiment, anesthetized with isofluorane (5% v/v) and euthanized by extracting blood from the descending aorta with a heparinized syringe to cold tubes and placed in ice until processing.

2.4. Growth Rate and Fecal Fat Extraction

Food consumption was measured daily and body-weight once per week. The growth or conversion curves were individually made relating diet consumption (g) to body-weight gain (g). Apparent dietary digestibility was calculated according to the following formula: (feed intake (g)–fecal weight (g))/feed intake (g). Fecal fat was extracted and weighed according to Macho-González et al. [22].

2.5. Glycemia

Blood samples were obtained under fasting conditions in the middle of the 3rd week to confirm the diabetic state, and at the end of the eighth week of the experiment. Blood samples were placed in heparinized tubes and subsequently centrifuged to obtain plasma at 986× g for 10 min. Immediately, glycemia was quantified using a plate reader (SPECTROstar Nano, BMG LABTECH, Offenburg, Germany) at 492 nm, using the GOD kit (Spinreact, Barcelona, Spain) [21].

2.6. Lipoprotein Isolation

Plasma was separated from the whole blood by centrifugation for 20 min at 615× g and stored at 4 °C until lipoprotein isolation. The different lipoprotein fractions were obtained from 2 mL plasma by saline gradient ultracentrifugation (Beckman L8-70M) using an SW-40.1 rotor following a modification of the Terpstra et al. method [31], according to Olivero-David et al. [11]. Briefly, the tubes were centrifuged for 21 h 40 min at 272,000× g (40,000 rpm) at 4 °C. Isolation of the lipoprotein fractions was performed taking into account conventional density range for rats of the different lipoprotein classes (VLDL (ρ_{20} < 1.0063 g/mL), IDL (1.0063 < ρ_{20} < 1.019), LDL (1.019 < ρ_{20} < 1.057), and HDL (1.057 < ρ_{20} <1.21 g/mL)) [31].

2.7. Plasma Lipid Analysis and Lipoprotein Composition

Triglycerides, total cholesterol and phospholipids were quantified in plasma and lipoprotein fractions (VLDL, IDL, LDL, and HDL). The measurements were made in plate readers at 492 nm (SPECTROstar Nano, BMG LABTECH, Offenburg, Germany), using the Triglycerides-LQ, Cholesterol-LQ and Phospholipids kits (Spinreact, Barcelona, Spain) according to the manufacturer's instructions. Total lipids were calculated as the sum of triglycerides, cholesterol and phospholipids [9,13]. The protein content

of isolated lipoproteins was determined by the Bradford method [32]. The total mass of each lipoprotein fraction was calculated as the sum of total lipids plus proteins (both in mg/dL). The atherogenic index (AI) was determined as follows: (total cholesterol–HDL cholesterol)/HDL cholesterol [9,13].

2.8. Arylesterase Activity Measurement

Rat plasma AE activity was measured using simulated body fluid (SBF) as a buffer according to Nus et al. [33]. One unit of AE was defined as the phenol (mmol) formed from phenylacetate per minute. Reaction rates were monitored at 270 nm in thermostated quartz cuvettes with a 10 mm light path, using a spectrophotometer (SPECTROstar Nano). Liver AE was measured in liver extracts according to Garcimartín et al. [9] and results expressed in AE units/mg protein. Liver protein was measured according to Bradford method [32]. A blank of each sample without plasma or liver extract was made to correct the spontaneous hydrolysis of phenylacetate in SBF. Each measurement was performed in duplicate.

2.9. VLDL and Liver Oxidation (TBARS Assay)

VLDL and liver oxidation (VLDL-ox and liver-ox, respectively) were quantified as malondialdehyde while thiobarbituric acid reactive substances (TBARS) content was measured in accordance to the Mihara and Uchiyama method [34] as indicated by Garcimartín et al. [9].

2.10. LDL-Receptor Levels by Western Blotting and Immunohistochemistry

LDL-receptor (*Ldlr*) levels were analyzed in liver by western blot. Samples were homogenized with lysis buffer containing 10 mM Tris-HCl (pH 7.4), 1% SDS, 1 mM sodium vanadate and 0.01% protease inhibitor cocktail (all from Sigma-Aldrich). Liver protein was measured using the Lowry method [35]. Equal amounts of protein (30 µg) were separated at 150 V in 8% (*v*/*v*) polyacrylamide gel (SDS–PAGE) and, after migration, transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Madrid, Spain) with Trans-Blot Turbo (Bio-Rad, Madrid, Spain) for 20 min and 2.5 mA. The membranes were blocked with 5% bovine serum albumin (BSA) for 1 h, and incubated overnight at 4 °C with primary antibodies anti-*Ldlr* (1:750) (sc-18823, Santa Cruz Biotechnology, Quimigen, Madrid, Spain) and anti-β-actin (1:30,000) (Sigma-Aldrich). Hybridization of antibodies was revealed by incubating the membranes with the appropriate secondary antibodies conjugated with peroxidase for 1h at room temperature. The chemiluminescence signal was detected using the ECL kit Select-kit (GE Healthcare, Madrid, Spain) and read in an ImageQuant LAS 500 (GE Healthcare, Madrid, Spain). Band-density was quantified using the Quantity One v.4.2.6 program and quantification was calculated referring to β-actin used as the loading control.

Paraffin-embedded liver sections were deparaffinized and rehydrated in a graded ethanol series. After retrieving citrate antigen and quenching endogenous peroxidase, sections were incubated with different primary antibodies overnight at 4 °C to determine the presence of *Ldlr* (Santa Cruz Biotechnology). The color reaction was developed with a polymerized horseradish peroxidase-conjugated secondary antibody and counterstained with hematoxylin. Fiji ImageJ processing software (U.S. National Institutes of Health, Bethesda, MD, USA) was used to count the stained vs. unstained pixels in each section. The immunolocalization of these proteins was studied by triplicate in eight different representative liver sections.

2.11. Statistical Analyses

Statistical analysis was performed using SPSS version 25.0 (SPSS Inc., Chicago, IL, USA). Results were expressed as mean ± SD. The effect of each independent variable was analyzed by means of an analysis of the variance (ANOVA) followed by T2-Tamhane or Bonferroni post-hoc test after assuming inequality or equality of variances, respectively. Differences in growth rate induced by diet were assessed by the ANCOVA test. The Chi-squared test was used to compare hypercholesterolemic or diabetic rat-distributions among groups. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Growth Rate, Feed Consumption and Fecal Excretion

Figure 1a–c shows the relationship between food intake and body-weight gain and the intercepts, slopes and significances found for the different groups. Diet significantly affected the growth curve. A significantly lower growth rate in D animals compared to ED and DE (ANCOVA, $p < 0.05$) was found

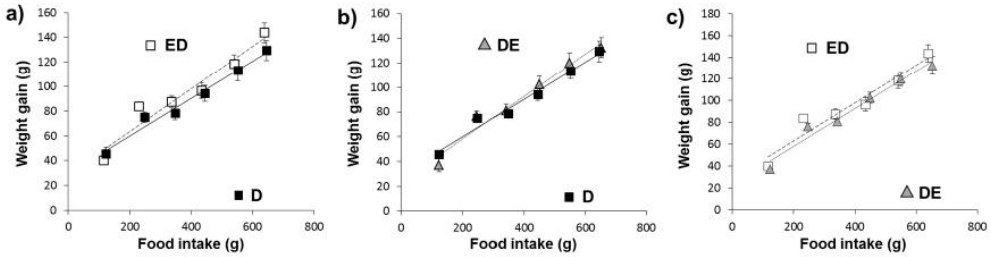

Figure 1. Growth rates of animals fed different diets. ED: Rats fed the Carob fruit extract (CFE)-diet since the beginning of the study; D: Rats fed the Chol-diet; DE: Rats fed the CFE-diet when the diabetic state was confirmed. For more abbreviations see footnotes of Table 1. Y = (slope with their SEM) * X + (intercept with their SEM), where Y is the body-weight gain and X is the food consumption. (**a**) ED [Y = (0.1789 ± 0.011) * X + (25.99 ± 1.66)] vs. D [Y = (0.149±0.004) * X + (26.98±2.10)] ANCOVA test $p < 0.05$; (**b**) DE [Y = (0.177 ± 0.005) * X + (23.76±1.92)] vs. D [Y = (0.149±0.004) * X + (26.98±2.10)] ANCOVA test $p < 0.05$; (**c**) ED [Y = (0.1789 ± 0.011) * X + (25.99 ± 1.66)] vs. DE [Y = (0.177 ± 0.005) * X + (23.76 ± 1.92)] ANCOVA test $p > 0.05$. R^2 adjust for ED, D and DE were 0.94, 0.95 and 0.95, respectively.

Table 2 summarizes the feed intake, growth rate, final weight and fecal excretions in the different rat groups. Fecal excretion, fecal moisture, fecal fat and dietary digestibility were significantly modified by diet (ANOVA at least, $p = 0.004$). Cholesterol intake was similar in all groups. Fecal excretion and moisture were significantly higher in ED and DE groups in comparison with D ($p < 0.001$). ED and DE vs. D rats showed a greater fecal fat amount but a lower food digestibility ($p < 0.001$). Non-significant differences ($p > 0.05$) were observed between ED group vs. DE group.

Table 2. Feed intake, anthropometric characteristics and fecal excretion of animals fed the experimental diets.

				ANOVA
	ED	**D**	**DE**	***p***
Cholesterol intake (g/wk)	1.07 ± 0.04	1.08 ± 0.08	1.09 ± 0.08	$p > 0.05$
Growth rate *	0.18 ± 0.03 ^{b}	0.15 ± 0.01 ^{a}	0.18 ± 0.01 ^{b}	$p = 0.019$
Final weight (g)	380.1 ± 39.3	366.6 ± 32.3	380.4 ± 22.3	$p > 0.05$
Fecal excretion (g/wk) **	14.10 ± 1.43 ^{b}	11.16 ± 0.43 ^{a}	13.57 ± 1.69 ^{b}	$p = 0.001$
Fecal moisture (%)	18.56 ± 1.55 ^{b}	14.04 ± 1.66 ^{a}	16.62 ± 1.40 ^{b}	$p < 0.001$
Fecal fat (mg/g feces) **	226.6 ± 26.93 ^{b}	170.6 ± 10.07 ^{a}	236.5 ± 25.20 ^{b}	$p < 0.001$
Dietary digestibility ***	0.85 ± 0.01 ^{a}	0.88 ± 0.01 ^{b}	0.86 ± 0.02 ^{ab}	$p = 0.004$

Results are means ± SD ($n = 8$). Labeled means in a row without a common letter differ ($p < 0.05$; a < b < c; ab = a or b; ANOVA followed by the Bonferroni or T2 Tamhane *post hoc* test). ED: rats fed the CFE-diet since the beginning of the study; D: rats fed the Chol-diet; DE: rats fed the CFE-diet when the diabetic state was confirmed. For more abbreviations see footnotes of Table 1. * Growth rate: Conversion rate relating food consumption (g) to body-weight gain (g). ** Data are dry matter weights; *** Dietary digestibility = (feed intake−feces)/feed intake.

3.2. Plasma Glucose and Lipid Concentrations

Table 3 shows glycemia, plasma lipid concentrations, the AI and the cholesterol-to-phospholipid and cholesterol-to-HDL cholesterol ratios in the different groups. Except for glycemia at 3rd week,

plasma phospholipids, and the cholesterol/phospholipid ratio; all other markers were significantly affected by diet (ANOVA at least $p = 0.013$).

Table 3. Plasma lipids, cholesterol: phospholipid and cholesterol: HDL-cholesterol ratio and atherogenic index of animals fed the experimental diets.

				ANOVA
	ED	**D**	**DE**	***p***
Glycemia 3rd week (mmol/L)	8.02 ± 0.57	8.04 ± 0.96	8.27 ± 0.70	$p > 0.05$
Glycemia 8th week (mmol/L)	15.76 ± 0.76 ^{b}	18.11 ± 1.65 ^{c}	13.94 ± 1.13 ^{a}	$p < 0.001$
Total cholesterol (mmol/L)	2.26 ± 0.12 ^{a}	2.95 ± 0.22 ^{c}	2.57 ± 0.16 ^{b}	$p < 0.001$
Triglycerides (mmol/L)	0.74 ± 0.05 ^{a}	0.87 ± 0.11 ^{b}	0.71 ± 0.13 ^{ab}	$p = 0.013$
Phospholipids (mmol/L)	1.14 ± 0.13	1.28 ± 0.09	1.26 ± 0.17	$p > 0.05$
Total lipids (mg/dL) *	235 ± 14.1 ^{a}	282 ± 29.8 ^{b}	252 ± 24.6 ^{ab}	$p = 0.003$
Cholesterol:phospholipids (mol/mol)	1.01 ± 0.09	1.13 ± 0.10	1.02 ± 0.16	$p > 0.05$
Cholesterol:HDL cholesterol (mol/mol)	1.65 ± 0.29 ^{a}	2.37 ± 0.45 ^{b}	2.10 ± 0.18 ^{b}	$p < 0.001$
Atherogenic index **	0.65 ± 0.29 ^{a}	1.37 ± 0.45 ^{b}	1.10 ± 0.18 ^{b}	$p < 0.001$

Results are means ± SD ($n = 8$). Labeled means in a row without a common letter differ ($p < 0.05$; a < b < c; ab = a or b; ANOVA followed by the Bonferroni or T2 Tamhane *post hoc* test). ED: rats fed the CFE-diet since the beginning of the study; D: rats fed the Chol-diet; DE: rats fed the CFE-diet when the diabetic state was confirmed. For more abbreviations see footnotes of Table 1. * Total lipids: cholesterol + triglycerides + phospholipids; ** Atherogenic index = (total cholesterol–HDL cholesterol)/HDL cholesterol. To transform mmol/L into mg/dL of cholesterol, triglycerides, and phospholipids, multiply data by 38.68, 89.0, and 75.0, respectively.

Hyperglycemia ($\geq$ 7 mmol/L) was found in 87.5% of ED and D rats, and 100% in DE rats at the 3rd week (chi square test $p < 0.001$). Marked hyperglycemia (>11.1 mmol/L) was found in all rats at the 8th week; however, ED and DE rats revealed significant lower glycemia than D rats ($p < 0.001$). DE rats showed significantly less final glycemia than ED rats ($p < 0.05$). D rats exhibited higher cholesterol than ED and DE ($p < 0.001$) and higher triglycerides than ED ($p = 0.013$). Hypercholesterolemia (total cholesterol $\geq$ 2.59 mmol/L) was found in 87.5% of D rats, 25% of DE rats but 0% of ED animals (chi square test $p < 0.001$). A higher cholesterol, AI, and cholesterol/HDL cholesterol ratio was found in DE rats vs. ED rats ($p < 0.05$).

3.3. Lipoprotein Composition

The composition of the different lipoprotein fractions in absolute values is shown in Table 4. Except for HDL-cholesterol and VLDL-total lipids most lipid components and total mass of VLDL, IDL, LDL and HDL fractions were significantly affected by diet (ANOVA $p < 0.001$). IDL proteins were affected by diet ($p = 0.005$).

ED rats show a significantly (at least $p < 0.05$) lower content of cholesterol, phospholipids and total mass in all lipoprotein fractions except HDL and higher triglycerides in VLDL, lower total lipids in IDL and LDL, and lower protein in IDL than their D counterparts. DE rats showed significantly (at least $p < 0.05$) lower cholesterol in VLDL, lower cholesterol and phospholipids but higher triglycerides in IDL, lower cholesterol, phospholipids, total lipids and total mass in LDL than their D counterparts.

VLDL fraction showed more cholesterol and phospholipids but fewer triglycerides in DE vs. ED rats. IDL fraction exhibited more triglycerides, phospholipids, total lipids, proteins and total mass in DE vs. ED rats. LDL fraction presented higher cholesterol, total lipids, and total mass in DE vs. ED rats. HDL fraction showed fewer phospholipids, total lipids and total mass in DE vs. ED rats.

Table 4. Lipoprotein component concentrations in plasma of animals fed the experimental diets.

				ANOVA
Lipoprotein	**ED**	**D**	**DE**	***p***
Cholesterol, mmol/L				
VLDL	0.22 ± 0.03 ^{a}	0.78 ± 0.09 ^{c}	0.62 ± 0.12 ^{b}	$p < 0.001$
IDL	0.44 ± 0.08 ^{a}	0.55 ± 0.03 ^{b}	0.41 ± 0.05 ^{a}	$p < 0.001$
LDL	0.16 ± 0.03 ^{a}	0.34 ± 0.06 ^{c}	0.24 ± 0.01 ^{b}	$p < 0.001$
HDL	1.40 ± 0.16	1.28 ± 0.19	1.22 ± 0.08	$p > 0.05$
Triglycerides, mmol/L				
VLDL	0.48 ± 0.07 ^{b}	0.30 ± 0.07 ^{a}	0.32 ± 0.08 ^{a}	$p < 0.001$
IDL	0.07 ± 0.02 ^{a}	0.13 ± 0.02 ^{b}	0.14 ± 0.05 ^{b}	$p < 0.001$
LDL	0.02 ± 0.01 ^{a}	0.07 ± 0.02 ^{c}	0.05 ± 0.01 ^{b}	$p < 0.001$
HDL	0.15 ± 0.01 ^{a}	0.23 ± 0.02 ^{b}	0.17 ± 0.03 ^{a}	$p < 0.001$
Phospholipids, mmol/L				
VLDL	0.25 ± 0.03 ^{a}	0.31 ± 0.03 ^{b}	0.31 ± 0.03 ^{b}	$p = 0.001$
IDL	0.12 ± 0.03 ^{a}	0.23 ± 0.01 ^{c}	0.18 ± 0.04 ^{b}	$p < 0.001$
LDL	0.07 ± 0.01 ^{a}	0.11 ± 0.01 ^{b}	0.08 ± 0.02 ^{a}	$p < 0.001$
HDL	0.72 ± 0.02 ^{c}	0.64 ± 0.06 ^{b}	0.52 ± 0.04 ^{a}	$p < 0.001$
Total lipids *, mg/dL				
VLDL	69.72 ± 7.67	79.50 ± 7.13	73.54 ± 8.67	$p > 0.05$
IDL	31.32 ± 4.69 ^{a}	48.41 ± 2.57 ^{c}	41.35 ± 4.28 ^{b}	$p < 0.001$
LDL	13.57 ± 2.14 ^{a}	27.70 ± 4.01 ^{c}	19.24 ± 1.25 ^{b}	$p < 0.001$
HDL	119.54 ± 5.82 ^{b}	115.94 ± 11.30 ^{b}	99.47 ± 7.11 ^{a}	$p < 0.001$
Proteins, mg/dL				
VLDL	5.43 ± 0.83	5.15 ± 0.86	4.89 ± 0.52	$p > 0.05$
IDL	6.31 ± 2.20 ^{a}	8.93 ± 1.32 ^{b}	8.26 ± 0.96 ^{ab}	$p = 0.005$
LDL	3.64 ± 1.34	4.51 ± 0.93	4.27 ± 0.76	$p > 0.05$
HDL	50.8 ± 8.94	48.8 ± 5.80	49.3 ± 5.79	$p > 0.05$
Total mass **, mg/dL				
VLDL	75.15 ± 6.33 ^{a}	84.65 ± 6.36 ^{b}	78.43 ± 8.62 ^{ab}	$p = 0.045$
IDL	37.63 ± 6.48 ^{a}	57.34 ± 3.13 ^{c}	49.61 ± 4.73 ^{b}	$p < 0.001$
LDL	17.21 ± 2.92 ^{a}	32.22 ± 4.32 ^{c}	23.51 ± 1.36 ^{b}	$p < 0.001$
HDL	170.3 ± 12.19 ^{b}	164.7 ± 14.92 ^{ab}	148.8 ± 7.54 ^{a}	$p < 0.001$

Results are means ± SD ($n = 8$). Labeled means in a row without a common letter differ ($p < 0.05$; a < b < c; ab = a or b; ANOVA followed by the Bonferroni or T2 Tamhane *post hoc* test). ED: rats fed the CFE-diet since the beginning of the study; D: rats fed the Chol-diet; DE: rats fed the CFE-diet when the diabetic state was confirmed. VLDL, very low-density lipoproteins; IDL, intermediate-density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins. For more abbreviations see footnotes of Table 1. * Total lipids: cholesterol + triglycerides + phospholipids; ** Total mass: total lipids + proteins.

3.4. Percentage Contribution of Lipids to Lipoprotein Composition

Figure 2 shows the percentage contribution of lipids and proteins to the total mass of plasma VLDL, IDL, LDL, and HDL fractions. Diet significantly affected all compositions of VLDL and IDL except proteins in both lipoproteins (ANOVA at least, $p = 0.031$); all LDL components (ANOVA at least, $p < 0.013$), and HDL triglycerides and phospholipids ($p < 0.001$).

ED rats had less cholesterol but more triglycerides (in percentage) in VLDL fraction than D rats ($p < 0.001$). Cholesterol contributed more while phospholipids less to the IDL total mass in ED rats vs. D rats. Cholesterol and triglycerides contributed less but phospholipids and proteins more to the total mass of LDL in ED vs. D rats. Triglycerides contributed less to the HDL fraction in ED vs. D rats.

DE rats show similar VLDL component contributions to total mass than D rats. Total triglycerides contributed more to total mass of IDL in DE vs. D rats. DE rats exhibited more proteins to LDL fraction than D groups. DE rats showed lower triglycerides and phospholipids contribution to the total HDL mass than D rats.

Cholesterol and phospholipids contributed more and triglyceride less to VLDL total mass in the DE vs ED rats. Cholesterol contributed less but triglycerides more to IDL particles in the DE vs. ED rats. Triglycerides and phospholipids contributed more to the LDL particles in the DE vs. ED rats. Triglycerides contributed more but phospholipids less to the HDL total mass in the DE vs. ED rats.

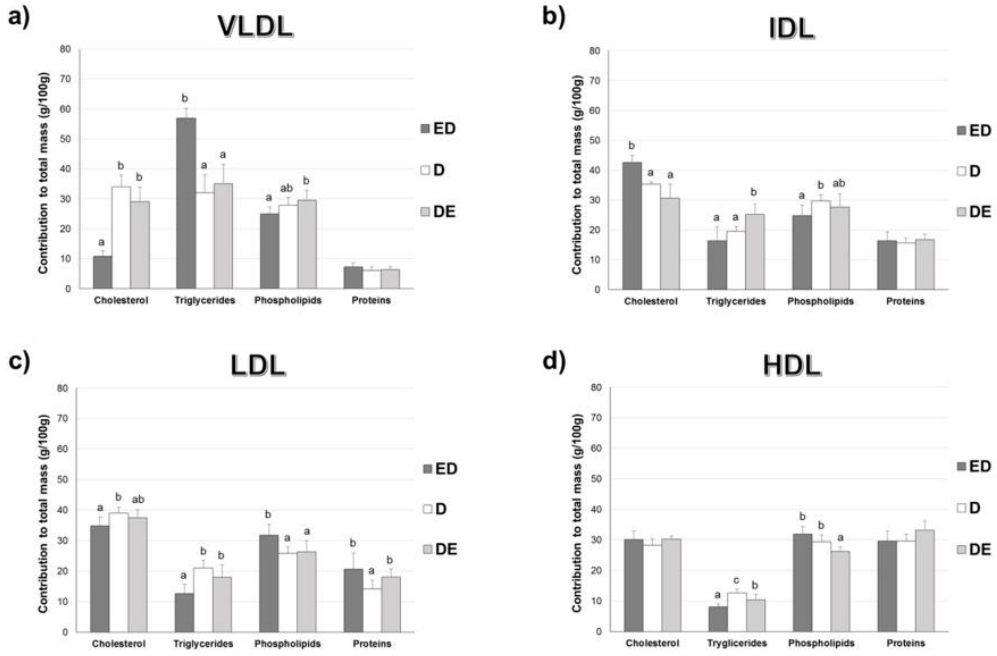

Figure 2. Percentage contribution (%) of proteins and the different lipids to the total mass of plasma VLDL, IDL, LDL and HDL fractions. Results are means ± SD ($n = 8$). ED: rats fed the CFE-diet since the beginning of the study; D: rats fed the Chol-diet; DE: rats fed the CFE-diet when the diabetic state was confirmed. For more abbreviations see footnotes of Table 1. (**a**) VLDL fraction composition: cholesterol ($p < 0.001$), triglycerides ($p < 0.001$), phospholipids ($p = 0.010$) and proteins ($p > 0.05$); (**b**) IDL fraction composition: cholesterol ($p < 0.001$), triglycerides ($p = 0.009$), phospholipids ($p = 0.031$) and proteins ($p > 0.05$); (**c**) LDL fraction composition: cholesterol ($p = 0.009$), triglycerides ($p < 0.001$), phospholipids ($p = 0.013$) and proteins ($p < 0.08$). (**d**) HDL fraction composition: cholesterol ($p > 0.05$), triglycerides ($p < 0.001$), phospholipids ($p < 0.001$) and proteins ($p > 0.05$). Labeled means for a variable without a common letter differ ($p < 0.05$; a < b < c; ab = a or b; ANOVA followed by the *post hoc* Bonferroni or T2 Tamhane test).

3.5. Arylesterase Activity, VLDL and Liver Oxidation

Plasma AE activity, the AE activity-to-plasma cholesterol ratio, liver AE activity, VLDL-ox and liver-ox are shown in Table 5. Diet significantly affected (ANOVA at least, $p = 0.024$) all parameters. Except for VLDL-ox, D rats showed lower plasma and liver AE, plasma AE/cholesterol ratio and higher liver-ox compared to ED and DE ones ($p < 0.05$). ED and DE showed higher plasma AE activity, plasma AE/cholesterol ratio, liver AE activity but lower liver oxidation respect to D ($p < 0.001$). Likewise, a significantly higher plasma AE activity and in the plasma AE/cholesterol ratio ($p < 0.001$) and lower VLDL-ox ($p < 0.05$) were observed in DE compared to D animals.

3.6. Liver Macroscopic Aspect

Liver hypertrophy and steatosis was clearly observed. However, ED rats followed by DE animals display a considerably steatosis reduction in macroscopic observation (Supplementary Figure S1).

Table 5. Plasma and liver arylesterase (AE) activity and VLDL and liver oxidation of animals fed the experimental diets.

				ANOVA
	ED	D	DE	*p*
Plasma AE (U/L) *	237 ± 43.06 ^{b}	163 ± 29.56 ^{a}	368 ± 58.59 ^{c}	$p < 0.001$
Plasma AE:cholesterol (U/mg) **	0.29 ± 0.05 ^{b}	0.15 ± 0.02 ^{a}	0.39 ± 0.05 ^{c}	$p < 0.001$
Liver AE (U/g protein)	7.70 ± 0.97 ^{b}	5.36 ± 0.83 ^{a}	10.93 ± 3.28 ^{b}	$p < 0.001$
VLDL-ox (TBARS, mg MDA/L)	2.31 ± 0.29 ^{ab}	2.58 ± 0.16 ^{b}	2.22 ± 0.23 ^{a}	$p = 0.024$
Liver-ox (TBARS, mg MDA/mg protein)	2.63 ± 0.58 ^{a}	3.63 ± 0.25 ^{b}	2.49 ± 0.33 ^{a}	$p < 0.001$

Results are means ± SD ($n = 8$). Labeled means in a row without a common letter differ ($p < 0.05$; a < b < c; ab = a or b; ANOVA followed by the Bonferroni or T2 Tamhane *post hoc* test). ED: rats fed the CFE-diet since the beginning of the study; D: rats fed the Chol-diet; DE: rats fed the CFE-diet when the diabetic state was confirmed. For more abbreviations see footnotes of Table 1. * One unit of AE was defined as mmols of phenol formed from phenylacetate per minute; ** Units AE (U/L)/total cholesterol (mg/L).

3.7. Liver LDL Receptor Levels

Diet significantly affected (ANOVA, $p < 0.001$) the hepatic *Ldlr* levels. ED and DE rats showed higher levels by Western blot of the *Ldlr* compared to D rats (Figure 3a). Non-significant differences ($p > 0.05$) were observed between ED group vs. DE group. Figure 3b,c show the immunohistochemistry data of *Ldlr* in liver sections. All groups displayed different *Ldlr* immunohistochemical staining levels (ANOVA, $p < 0.001$). ED and DE rats exhibited significantly higher *Ldlr* levels than D counterparts ($p < 0.05$).

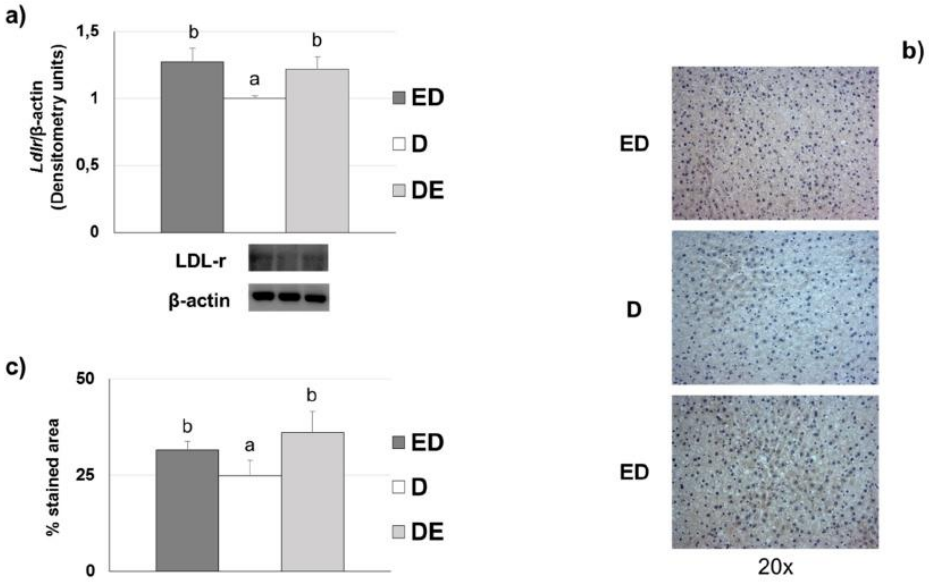

Figure 3. *Ldlr* levels liver localization in the different diabetic groups fed the three experimental diets ($n = 8$ rats/group). ED: rats fed the CFE-diet since the beginning of the study; D: rats fed the Chol-diet; DE: rats fed the CFE-diet when the diabetic state was confirmed. For more abbreviations see footnotes of Table 1. (**a**) Representative blots from liver *Ldlr* levels. Analysis of the variance (ANOVA), $p < 0.001$. Labeled means for a variable without a common letter differ ($p < 0.05$; a < b; ANOVA followed by the *post hoc* T2 Tamhane test). (**b**) The immunolocalization of *Ldlr* was studied by triplicate in eight different representative liver sections (20×). (**c**) Percentage stained area of hepatocytes *Ldlr*. Analysis of the variance (ANOVA), $p < 0.001$. Labeled means for a variable without a common letter differ ($p < 0.05$; a < b; ANOVA followed by the *post hoc* Bonferroni test).

4. Discussion

The results described show the effect of CFE included in a meat matrix as a nutritional strategy to reduce the qualitative and quantitative lipoprotein alterations in T2DM rats fed a high saturated-fat/high-cholesterol diet. CFE prevented the negative effects of this atherogenic diet by reducing basal glycemia, cholesterolemia, triglyceridemia and liver oxidation, at the same time as improving the lipoprotein profile [11,36] and increasing the AE activity, fecal fat excretion and *Ldlr* levels in STZ-NAD diabetic rats. These results are relevant given the concern between high meat-consumption and degenerative diseases prevalence [1], although it has been observed that meat-product consumption can improve GLP-1 and GPI, incretins that are affected in T2DM subjects [4,6,7]. Therefore, the possibility of reducing the negative effects high consumption of meat products in the frame of an atherogenic diet by including the CFE as a functional ingredient in the meat product can be suggested.

Based on the evidence of the present and previous related studies, a mechanism explaining results for CFE-RM consumption in STZ-NAD diabetic rats is proposed (Figure 4).

All study diets were well accepted, as observed in the intake and growth data, which are consistent with results of our group studying different RM on cholesterol fed-rats [10–12]. Although the final weights of the study animals were very similar among all groups, a lower growth slope in D rats was found, which is typical of animals fed cholesterol-enriched diets [9,13,30,37]. This growth slope was higher in the ED and DE groups. On the other hand, CFE diet induced greater fecal excretion and higher fecal fat content with respect to rats fed the control-RM diet, giving rise to dietary digestibility reduction. This effect is justified because CFE-composition is based on insoluble fiber and proanthocyanidins, which leads to a reduction in fat and carbohydrate digestions, as observed by Macho-González et al. in postprandial studies developed in healthy rats fed the same extract [21,22]. Despite the higher fecal excretions of ED and DE rats with respect to D animals, the final body weight tended (non-significantly; $p > 0.05$) to increase, suggesting that CFE-fed rat release less fatty acids from adipose tissue, improving the diabetogenic situation induced by STZ-NAD administration [6,7].

One of the most stressful findings of the present paper is the significant reduction in fasting glycemia observed in STZ-NAD animals fed with the CFE (13% in ED rats and 23% in DE rats) vs. the D counterparts. As discussed, CFE was able to reduce postprandial glycemia in chow-diet rats [21]. Some fiber compounds have been found to reduce glycemia and lipemia [38]. Cholesterol-enriched diets induced moderate hypercholesterolemia in group D (87.5% of D rats show cholesterol levels $\geq$ 2.59 mmol/L or $\geq$ 100 mg/dL, defined as cut-off point) but 0% in ED and only 25% of DE rats displayed this level, suggesting the hypocholesterolemic effect of the CFE amount assayed [36]. These animals also show a modified lipoprotein profile according to the AI and the cholesterol/phospholipids and cholesterol/HDL-cholesterol ratios that were in line with previous results obtained by our group [9,13] allowing us to agree with Ruiz-Roso et al. [39] who showed that carob fruit reduced plasma cholesterol in hypercholesterolemic subjects. Results on lipoprotein fractions indicate that D animals showed the typical lipoprotein profile of hypercholesterolemic rats with the presence of cholesterol-enriched and triglyceride-depleted VLDL (β-VLDL) [11–13,40,41]. In contrast, the ED group displayed more normalized, triglyceride-enriched VLDL. These β-VLDL have been defined as atherogenic lipoproteins for the rat [42]. In addition, the presence of increased IDL and LDL particles was also clearly observed. According to Sánchez-Muniz et al., as each LDL particle contains only one ApoB100 molecule, it can be calculated that ED presented 19% and DE 5% less average LDL particles than D counterparts [43]. The LDL levels in D rats also correspond to the hypercholesterolemic diet effect itself, as observed in previous studies by our group [9–11]. These LDL values also seem related to the higher triglyceride-availability in D rats, as well as a clear decrease in catabolism, as a consequence of a lower expression of the hepatic *Ldlr*, which implies a clear reduction of CVD risk by CFE-RM-consumption mainly in the ED group. In situations where *Ldlr* have downregulated, LDL-removal decreases and VLDL remnant-removal should also decrease. Conversion of VLDL to LDL would then increase. Both of these circumstances would also increase LDL concentrations [44]. The triglyceride contribution to HDL total mass was higher in D rats vs. ED and DE, partially due to

the higher levels of plasma triglycerides observed, that in turn favors the production of VLDL and promotes triglyceride enrichment of HDL particles [6]. The *Ldlr* reduction may also be a consequence of the greater synthesis of LDL associated with the hypercholesterolemic diets. The lesser liver *Ldlr* amount in D animals seems linked to a mechanism to avoid cholesterol-accumulation in the liver [9,13] but is also probably related to an insulin-resistance status in these rats, a fact observed in STZ-NAD animals fed a high-saturated diet [45]. Finally, D animals show higher VLDL-ox and liver-ox amounts while lower plasma and liver AE, in line with the reduced antioxidant defense observed in diabetic animals and patients [46].

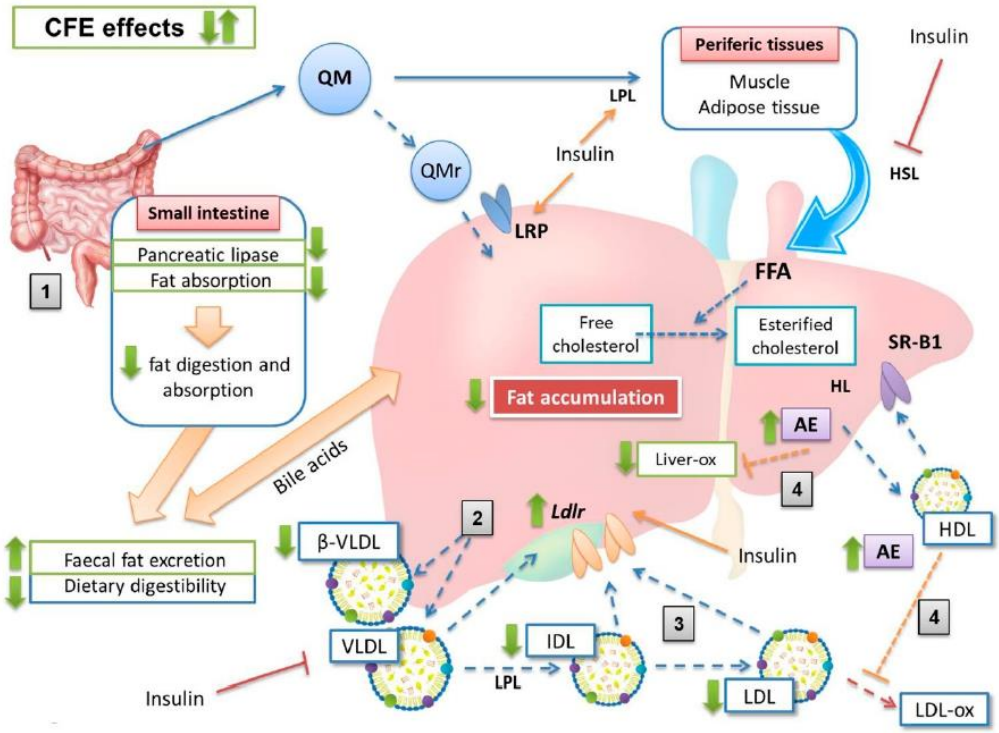

Figure 4. Proposed mechanism of the carob fruit extract (CFE) effects on lipoprotein metabolism. The diabetogenic status induced by STZ-NAD administration in addition to the atherogenic diet give raise to a hyperglycemic status compatible with a decrease in plasma insulin levels [25]. The insulinemia reduction implies higher FFA release increased VLDL production and *Ldlr* decrease [6]. The cholesterol-enriched diet produces increased level of cholesterol-enriched VLDL (β-VLDL) [9,13]. Green arrow indicates the increase (↑) or decrease (↓) for each marker assigned. Stages: (1) CFE increases the fat excretion of the atherogenic diet, mainly by decreasing dietary digestibility (reduction of fat digestion and absorption); (2) the lower plasma cholesterol and triglycerides in CFE groups correspond to a lower production of triglyceride enriched-VLDL and decreased β-VLDL synthesis; (3) CFE decreases the IDL and LDL levels, as a consequence of a higher hepatic *Ldlr* levels; (4) CFE increases the plasma and liver AE activities, reducing the VLDL and liver oxidation observed in the non-CFE rats. AE, arylesterase; CFE, carob fruit extract; FFA, free fatty acids; HDL, high-density lipoproteins; HL, hepatic lipase; HSL, hormone-sensitive lipase; IDL, intermediate-density lipoproteins; LDL, low-density lipoproteins; *Ldlr*, low density lipoprotein receptor; LRP, low density lipoprotein receptor-related protein; QM, chylomicrons; QMr, remnant chylomicrons; SR-B1, hepatic scavenger receptor B-I; VLDL, very low-density lipoproteins; β-VLDL, cholesterol-enriched very low-density lipoproteins.

As described, these alterations in the lipoprotein composition were partially reversed in the CFE-RM animals, mainly in ED rats that show lower cholesterol and more triglycerides contributing to

the VLDL total mass [10]. Likewise, it can be suggested that hepatic LDL and LDL re-uptake was more effectively performed in CFE-RM groups, basically due to the higher levels of *Ldlr* found. These results may be related to an increase in cholesterol esterification as a strategy to increase *Ldlr* levels [47], as well as to the possible higher plasma insulin values associated with proanthocyanidins-consumption [48]. However, CFE was not able to increase HDL levels. It has been proposed that in hypercholesterolemic rats the decrease in HDL levels is related to the uptake of these lipoproteins by hepatic scavenger receptor B-I (SR-BI) in order to increase biliary excretion and reduce plasma cholesterol levels [49].

The data of the present study suggest a greater VLDL oxidation and greater liver lipid peroxidation susceptibilities in D group, both associated with the consumption of hypercholesterolemic diet and the diabetic induction [9,50,51]. However, animals consuming CFE-RM showed reduction in VLDL and liver oxidations, as a consequence of the marked antioxidant properties of proanthocyanidins [52]. These results also seem be associated with the higher plasma and liver AE activities of these groups, suggesting that proanthocyanidin active metabolites could increase the antioxidant defense. In the same way, as serum paraoxonase 1 (PON1) is synthesized in the liver, the biggest changes associated with CFE-consumption was observed in this organ. Estrada-Luna et al. observed PON1 expression and activity increases in mice fed pomegranate juice in the frame of a high-fat diet [53]. Rock et al. also observed a PON1 activity increase in diabetic patients who ingested a pomegranate-rich tannin preparation [54]. Therefore, all these data taken together would justify a lower susceptibility to oxidation and a lower atherosclerosis risk in CFE groups [55,56].

Despite the positive results observed, this study presents some possible limitations: (1) only one dose of CFE was tested, (2) only one dose of STZ was employed, (3) the study lasted only eight weeks, (4) the study was performed only on growing male Wistar rats, and (5) rat lipoprotein profile was not assessed at the beginning of the study.

5. Conclusions

Results show for the first time that CFE-RM reduces the negative effects of an atherogenic diet, improving glycemia, lipemia, AE activity, *Ldlr* levels and lipoprotein profiles by reducing the presence of β-VLDL, IDL and LDL which leads to a reduction in CDV risk. Therefore, we can suggest that CFE is an adequate functional ingredient to be included in a meat matrix aimed to be preferably consumed by prediabetes and T2DM patients allowing them to get the high nutrient content of these food products and to correct lipoprotein alterations.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/11/2/332/s1, Table S1: Composition of the Restructured Meat (RM) incorporated into the experimental diets fed to male Wistar rats, Figure S1: Macroscopic aspect of a representative liver of the experimental groups.

Author Contributions: A.M.-G., A.G. and M.E.L.-O. have carried out the experiments, participated in data analysis, and drafted the manuscript. I.M.T. provided technical assistance in feces analysis. S.B. and B.R.-R. participated in the study design and manuscript review. F.J.S.-M. and J.B. are the guarantors of the article and collaborated in the design, discussion and writing of the paper. All authors read and approved the final manuscript.

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Abbreviations

AE, arylesterase; AI, atherogenic index; CDV, cardiovascular disease; CFE, carob fruit extract; HDL, high-density lipoprotein; IDL, intermediate-density lipoproteins; LDL, low-density lipoprotein; *Ldlr*, LDL receptor; NAD, nicotinamide; PON1, paraoxonase 1; RM, restructured meat; STZ, streptozotocin; T2DM, Type 2 Diabetes Mellitus; VLDL, very low-density lipoprotein.

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Publicación 9

Carob Fruit Extract-Enriched Meat improve pancreatic beta-cell dysfunction, hepatic insulin signaling and lipogenesis in late stage T2DM model

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Antecedentes: La DMT2 es una patología crónica caracterizada por RI e hiperglucemia. Sin embargo, el estadio de la enfermedad determina las características fisiopatológicas de la misma (Fonseca, 2009; Skovsø, 2014). La dieta es un factor central tanto en el desarrollo de la DMT2, como en el control de la misma. Concretamente, las PACs se han postulado como unos potentes agentes antidiabéticos, regulando el metabolismo de los hidratos de carbono, favoreciendo la síntesis y secreción de insulina y mejorando la señalización de insulina en los tejidos periféricos (Rauf y cols., 2019; Yang & Chan, 2017). El CFE, una fuente rica en fibra y PACs, ha demostrado efectos prometedores sobre el estatus postprandial (Artículos 1 y 2), así como sobre la dislipemia diabética (Artículo 8). No obstante, hasta la fecha no ha sido probado el impacto del consumo del CFE incluido en una matriz cárnica sobre la disfunción pancreática en un modelo de estado tardío de DMT2.

Hipótesis: El consumo de un cárnico enriquecido en CFE mejora la disfunción pancreática, la señalización de insulina y la lipogénesis *de novo* hepáticas en un modelo de DMT2 avanzada.

Resultados: El estudio histopatológico del páncreas reveló una marcada atrofia de los islotes de Langerhäns, con pequeñas vacuolas en las células acinares y niveles reducidos de insulina en el grupo D. Además, el hígado mostró un perfil hispopatológico compatible con NAFLD, con una elevada infiltración grasa. En cambio, los grupos que consumieron CFE como estrategia preventiva (ED) y terapéutica (DE) presentaron islotes de Langerhäns regulares, un elevado contenido de insulina y una mayor proliferación celular en comparación con el grupo D. A nivel hepático se encontró un incremento de los niveles y la fosforilación de las proteínas implicadas en la señalización de insulina InsR/PI3K/AKT/GSK3 que condujo a un mayor almacenamiento de glucógeno. Por último, el porcentaje de esteatosis fue significativamente menor en los grupos ED y DE, los cuales presentaron una marcada disminución de los factores de transcripción lipogénicos LXRα/β, SREBP-1c and ChREBP respecto al control.

Conclusiones: El consumo como estrategia preventiva o terapéutica mejora la sensibilidad a la insulina al reducir la disfunción pancreática y favorecer la regeneración de las células β pancreáticas, lo que indirectamente contribuye a una mejor homeostasis de la glucosa a través de la vía InsR/PI3K/AKT/GSK3. Asimismo, el CFE reduce la acumulación de lípidos hepáticos al regular a la baja los factores de transcripción lipogénicos LXRα/β, SREBP-1c and ChREBP. El consumo de carne funcional enriquecida en CFE parece ser una herramienta eficaz para mejorar la fisiopatología de la DMT2.

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Carob fruit extract-enriched meat improves pancreatic beta-cell dysfunction, hepatic insulin signaling and lipogenesis in late-stage type 2 diabetes mellitus model

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Abstract

The inclusion of functional bioactive compounds of dietary fiber in meat products has been demonstrated to exert a significant impact on human health. Carob fruit extract (CFE) is a dietary fiber rich in proanthocyanidins with known antioxidant, hypolipidemic and hypoglycemic effects. Consumption of CFE-enriched meat (CFE-RM) may provide interesting benefits in late-stage type 2 diabetes mellitus (T2DM). To explore the antidiabetic mechanisms of CFE-RM, we used a model of late-stage T2DM in Wistar rats fed a high-saturated-fat/high-cholesterol diet (Chol-diet) and injected streptozotocin plus nicotinamide (D group). The effects of CFE-RM were tested by incorporating it into the diet as preventive strategy (ED group) or curative treatment (DE group). CFE-RM had a positive effect on glycemia, enhancing hepatic insulin sensitivity and improving pancreatic β-cell regeneration in both ED and DE groups. Western blotting and immunohistochemistry suggested that CFE-RM increased levels of insulin receptor β and phosphatidylinositol-3-kinase, as well as the downstream target phospho-Akt (at Ser^{473}). CFE-RM also up-regulated glucose transporter 2, which improves the insulin-mediated glucose uptake by the liver, and promoted phosphorylation of glycogen synthesis kinase-3βprotein (at ser^{9}), consequently increasing the hepatic glycogen content. In addition, CFE-RM decreased fatty liver by suppressing *de novo* lipogenesis activation due to down-regulation of liver X receptor-α/β, sterol regulatory element binding protein-1c and carbohydrate-response element-binding protein transcription factors. Our findings suggest that the consumption of CFE-RM included in the diet as a functional food should be considered as a suitable nutritional strategy to prevent or manage late-stage T2DM.

Abbreviation: CFE, carob fruit extract; CFE-RM, CFE-enriched meat; CFE-Chol-diet, high-saturated-fat/high-cholesterol diet plus CFE-enriched meat; Chol-diet, high-saturated-fat/high-cholesterol diet; ChREBP, carbohydrate-response element-binding protein; DNL, *de novo* lipogenesis; GLUT2, glucose transporter 2; GSK-3β, glycogen synthesis kinase-3β; IHQ, immunohistochemistry; InsRβ, insulin receptor β; IR, insulin resistance; LXRαβ, liver X receptor-αβ; NAD, nicotinamide; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PAS, periodic acid-Schiff; PI3K, phosphatidylinositol-3-kinase; RM, restructured meat; SREBP-1c, sterol regulatory element binding protein-1c; STZ, streptozotocin; T2DM, type 2 diabetes mellitus; WB, Western blot

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1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease caused by insulin resistance (IR) and hyperglycemia. Although, at early stages, pancreatic β cells are able to counterbalance IR, increasing insulin production, the progression of the pathology causes pancreatic β-cell dysfunction. This late stage of T2DM is characterized by hyperglycemia along with low insulinemia [1]. The development and progress of T2DM result in triple interaction between multiple gene polymorphisms and environmental and lifestyle factors. Western diets characterized by high intake of red meat in the frame of inadequate diets with low complex cereals, vegetable and fruit content increased the risk of IR and T2DM [2]. Nutrition is very important in managing T2DM because both type and quantity of food impact on the hyperglycemia. In fact, the highest incidence of T2DM occurs in societies where consumption of energy-dense diets rich in saturated fatty acids is high, while dietary fiber intake remains low. Among the most consumed foods, meat has been subject of numerous studies and linked to well-known detrimental metabolic consequences. High meat intakes seem to promote IR and glucose intolerance in the early term, which later provoke obesity, T2DM and cardiovascular complications [2].

On the other hand, designing functional food with bioactive ingredients can help in T2DM prevention and/or management [3]. Taking advantage of the high acceptance of meat, the addition of functional ingredients to meat matrices could be a suitable alternative not only to avoid the potential negative effects associated to its consumption but also to promote additional health benefits. In this line, dietary fiber is one of the functional ingredients that can be incorporated to meat products. It is well known that polyphenol-rich dietary products improve carbohydrate and lipid metabolism; attenuate hyperglycemia, dyslipidemia and IR; and relieve oxidative stress and inflammatory processes by modulating several signaling pathways [4]. Within polyphenols, flavonoids stand out as promising functional ingredients with protective properties for T2DM [3].

Carob fruit extract (CFE) is an excellent source of soluble and insoluble fiber and contains a panel of polyphenols such as proanthocyanidins, gallic acid and its derivatives, as well as condensed tannins [5]. It has been previously described that colonic fermentation of nonextractable polyphenols obtained from carob pod produces absorbable metabolites with potential health benefits and high biological activity [6]. Previous results of our group demonstrated CFE's ability to reduce postprandial hyperglycemia [7] and hypertriglyceridemia [8] when administered in healthy rats. Lastly, we have also demonstrated the hypoglycemic effect of CFE-enriched meat in a rat model of T2DM induced by high-saturated-fat diet [9].

Taking all of these into account, we formulated a functional meat incorporating CFE as bioactive compound in order to study its use as preventive strategy and curative treatment in a late-stage T2DM rat model induced by a high-saturated-fat/high-cholesterol diet and one injection of streptozotocin-nicotinamide (STZ-NAD). First results of this experiment confirmed that CFE-enriched meat (CFE-RM) maintained the hypoglycemic and hypolipidemic properties previously reported [9]. Moreover, CFE-RM induced greater fecal fat excretion and improved the lipoprotein profile of diabetic rats [9]. However, the antidiabetic effect of CFE-RM has not been previously analyzed in late-stage T2DM. Thus, the present study was aimed to examine the potential preventive or curative antidiabetic properties of CFE-RM in STZ-NAD diabetic rats fed a high-saturated-fat/high-cholesterol diet.

2. Materials and methods

2.1. CFE, restructured meat and diet preparation

CFE is a natural insoluble dietary fiber with very high proanthocyanidins content. Carob tree (*Ceratonia siliqua L.*) gives pods. However, due to the sweet pulp content of pods, it is extended the expression carob fruit instead carob pod.

CFE was obtained from the carob pulp following the procedure described in WO2004/014150 patent [10]. Its average composition according to this patent was as follows: 4.5%–7% proteins, 0.5%–1% fats, 1.5%–3.5% sugars, 3%–4% ash (0.6%–1.1% calcium, 0.02%–0.026% sodium, 0.025%–0.047% potassium and 0.01%–0.016% iron) and 74%–84% total dietary fiber (with 1%–3% soluble fiber and 71%–81% insoluble fiber). Moreover, the polyphenols are composed of 34%–48% nonextractable PACs, and 0.5%–1% soluble extractable polyphenols have been previously indicated in Macho-González et al. [8]. Although the specific RMs formulation protocol, and RM and diet compositions have been previously detailed by Macho-Gonzalez et al. [9]; briefly, we report here that, for this experiment, two different restructured meats (RMs) were formulated: one denominated control-RM with 4 g/kg of cellulose in order to equilibrate the amount of fiber between the two RMs and the other one enriched with CFE (4 g/kg) designed as CFE-RM. Both RMs were included in a high-saturated-fat (20.4% of total energy) and high-cholesterol (nearly 10 g/kg) diet. Hence, there were two experimental diets: control diet (Chol-diet) with control-RM and CFE-Chol-diet with CFE-RM. The composition of these diets is detailed in Table 1.

2.2. Induction of T2DM and experimental design

All experiments were performed in compliance with Directive 86/609/EEC of 24 November 1986 (amended by Directive 2003/65/EEC of 22 July 2003) on the protection of scientific research animals. The study was approved by the Spanish Science and Technology Advisory Committee (project AGL2014-53207-C2-2-R) and by the Ethics Committee of the Universidad Complutense de Madrid (Spain). Twenty-four male Wistar rats aged 2 months were obtained from Harlan Laboratories models (Harlan S.L., Barcelona, Spain) and housed in couples under controlled temperature and light (22.3°C ±1.9°C and 12-h light/dark cycle, respectively) at the Centro de Experimentation Animal of the University of Alcalá, Madrid, Spain (registered number ES280050001165). Animals were pair-housed, and food and tap water were provided *ad libitum*. Powered diet was provided along the experiment. Powdered chow diet was introduced 2 weeks before the beginning of the experiment in order to become the rats used to eat the new formulation. Three different rat groups ($n=8$) were used: T2DM control group (D), CFE prevention group (ED) and CFE treatment group (DE). Fig. 1 summarizes the experimental design. Thus, the D and DE groups started eating Chol-

Table 1
Composition of the experimental diets fed to Wistar rats

Dietary components	Chol-diet	CFE-Chol-diet
Ingredients, g/kg		
Sucrose	68.25	68.25
Corn starch	275.73	275.73
Casein	94.25	94.25
Maltodextrin	94.25	94.25
Cellulose	48.86	48.86
PM 205B SAFE	50.05	50.05
PV 200 SAFE	7.15	7.15
Soybean oil	47.91	47.91
L-Cysteine	2.02	2.02
Cholesterol	9.1	9.1
Cholic acid	1.3	1.3
Freeze-dried restructured meat (RM)	301.14 [b]	301.14 [b]
Nutrient composition		
Protein, %	14.0	14.0
Fat, %	49.0	49.0
Cholesterol, g/kg	9.83	9.83
SFA:MUFA:PUFA ratio	2.10/2.32/1	2.10/2.32/1
Energy content,[a] MJ/kg	20.36	20.36

Chol-diet, diet containing the control-RM and 1.4% cholesterol and 0.2% cholic acid; CFE-Chol-diet, diet containing the CFE-RM and 1.4% cholesterol and 0.2% cholic acid.

[a] Data were calculated according to the energy equivalents for carbohydrate [16.73 kJ/g (4.0 kcal/g)], fat [37.65 kJ/g (9.0 kcal/g)] and protein [16.73 kJ/g (4.0 kcal/g)].

[b] Control-RM was formulated with cellulose (4 g/kg) and CFE-RM was formulated with CFE (4 g/kg) in order to maintain the same contribution of dietary fiber to diets.

diet during 3 weeks, while the ED group consumed CFE-Chol-diet during the entire experiment. At the third week of the study, diabetes was induced in all rats by intraperitoneal injection of STZ (65 mg/kg BW) and NAD (225 mg/kg BW) (Sigma-Aldrich, Madrid, Spain), and 72 h later, hyperglycemia was confirmed. D and ED kept eating Chol-diet and CFE-Chol-diet, respectively, for 5 weeks more. On the contrary, DE changed Chol-diet from this moment to CFE-Chol-diet until the end. Globally, the study lasted 8 weeks. At the end of experiment and to avoid interassay variations, all rats were overnight fasted and euthanized by blood extraction under anesthesia with isofluorane (5% vol/vol). Blood was collected from the descending aorta with a heparinized syringe in cold tubes and placed in ice until processing. Livers and pancreas were removed, weighed and ready to be processed later. Histopathology and immunohistochemistry (IHQ) studies were performed in liver and pancreas samples fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, dehydrated and embedded in paraffin, while Western blots (WBs) or biochemical determinations were done in samples that had been kept frozen at 80°C before analysis.

2.3. Determination of glycemia, insulinemia and HOMA-β index

In order to confirm the diabetic state, blood was taken from the tail at third and eighth experimental weeks. To obtain plasma, blood samples were placed in heparinized tubes and subsequently centrifuged at 986g for 10 min. Immediately, postprandial glucose was quantified at 492 nm in a plate reader (SPECTROstar Nano, BMG LABTECH, Offenburg, Germany) using the GOD kit (Spinreact, Barcelona, Spain). Plasma insulin concentration was measured using enzyme-linked immunoabsorbent assay (ELISA) (Rat insulin ELISA Kit, ELR-Insulin, RayBiotech, Inc., USA) according to the manufacturer's manual. Color intensity increase was evaluated at 450 nm in a plate reader. HOMA-β index was calculated by the formula 20 × fasting plasma insulin (μIU/ml)/fasting glucose (mmol/L) − 3.5 [11,12].

2.4. WB analysis

Western blotting method was carried out as previously described by Garcimartín et al. [13]. Livers were homogenized with lyses buffer, and equal amounts of proteins (DS protein assays, Biorad Spain) were separated in denaturing SDS 10% polyacrylamide gels. Membranes were incubated overnight at 4°C with the following primary antibodies: anti-insulin receptor β (InsRβ), anti-phosphatidylinositol-3-kinase (PI3K), anti-AKT2, anti-pAKT2^{ser473}, anti-glycogen synthesis kinase-3β (GSK3β), anti-pGSK3β^{ser9}, anti-glucose transporter 2 (GLUT2), anti-sterol regulatory element binding protein-1c (SREBP-1c) and anti-carbohydrate-response element-binding protein (CHREBP) (Santa Cruz Biotechnology, Quimigen, Spain). Anti-β-actin and/or anti-α-tubulin were used as the loading controls. Membranes were incubated with peroxide-conjugated secondary antibodies for 1 h at room temperature. The chemiluminescence signal was detected using the ECL kit Select-kit (GE Healthcare, Madrid, Spain) and read in an ImageQuant LAS 500 (GE Healthcare, Madrid, Spain). The quantification of the protein levels was made using Image Quant 5.0 software (GE Healthcare Life Sciences) and expressed as densitometry unit with respect to loading control.

2.5. Liver and pancreatic histopathology and IHQ

The liver and pancreas sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, dehydrated and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) and/or periodic acid-Schiff (PAS) reagent according to the routine methods. Images of the histological sections were obtained using ×4, ×10 and ×20 magnifications and were analyzed by using a digital camera (Leica DFC 320 camera, L'Hospitalet del Llobregat, Spain) attached to a light microscope (Leica DM LB2). The number of islets in each section of the pancreas and the area of the pancreatic islet (μm^2) were analyzed from H&E pancreas sections. Hepatic steatosis area (H&E sections) and glycogen area (PAS sections) were performed from 15 fields per liver section per rat and expressed as a percentage of the total surface per field by using Fiji ImageJ v1.52j software (National Institutes of Health: rsb.info.nih.gov/ij). With respect to IHQ, liver and pancreatic tissue sections were deparaffinized, and citrate antigen was recovered as well as endogenous peroxidase was quenched with 3% hydrogen peroxide. Then, the sections were incubated overnight at 4°C with the following primary polyclonal antibodies: anti-insulin, anti-PCNA, anti-InsRβ, anti-pAKT2^{ser473}, anti-pGSK3β^{ser9}, anti-liver X receptor-αβ (LXRα/β), anti-SREBP-1c and anti-CHREBP (Santa Cruz Biotechnology Quimigen, Madrid, Spain). After washes, the sections were covered with the appropriated biotinylated secondary antibody. Immunochemical staining was performed by using streptavidin–biotin-conjugated horseradish peroxidase (Sigma Aldrich, Madrid, Spain) and visualized by incubation with 3,3′-diaminobenzidine (DAB) (Sigma Aldrich, Madrid, Spain). The sections were counterstained by Harris' hematoxylin, dehydrated and mounted. A total of 15 fields per section per rat (×400 magnifications for image analysis) were selected and analyzed. The relative pancreatic β-cell islet density was calculated as the number of immune-positive insulin cells with respect to the total number cells per islet in a total of 20 islets per group. The numbers of PCNA-positive (dark brown nuclear staining) and PCNA-negative (blue light) pancreatic islet nuclei were recorded for calculation of the PCNA labeling index. At least 1000 nuclei (endocrine cells) were analyzed for each animal. Hepatic insulin pathway markers and lipogenic transcriptional factors immunoreactivities were quantified using the percentage of positive DAB area, measured using the color deconvolution plugin from Fiji ImageJ v1.52j software. The background for the DAB staining in the IHC was minimized by adjusting the maximum threshold value without removing the true DAB signal. The maximum threshold was tested for at least five images to get an average maximum threshold value. In addition, the same procedure was performed for the stain-negative areas to quantify the background that was further subtracted.

2.6. Liver glycogen content

Liver glycogen was determined by a slight modification of the Morales et al. method [14]. Briefly, liver samples (500 mg) were digested, boiling them in 30% KOH for 20 min. After cooling, 3 ml of 95% ethanol and a drop of 1 M ammonium acetate were added and heated again until boiling. Tubes were cooled and centrifuged at 3000g for 10 min, and the supernatants were discarded. Residues were then washed three times, and 4 ml of anthrone reagent was added to 2 ml of sample and kept in ice. Afterward, tubes were incubated during 20 min at 100°C for color development, and absorbance was measured

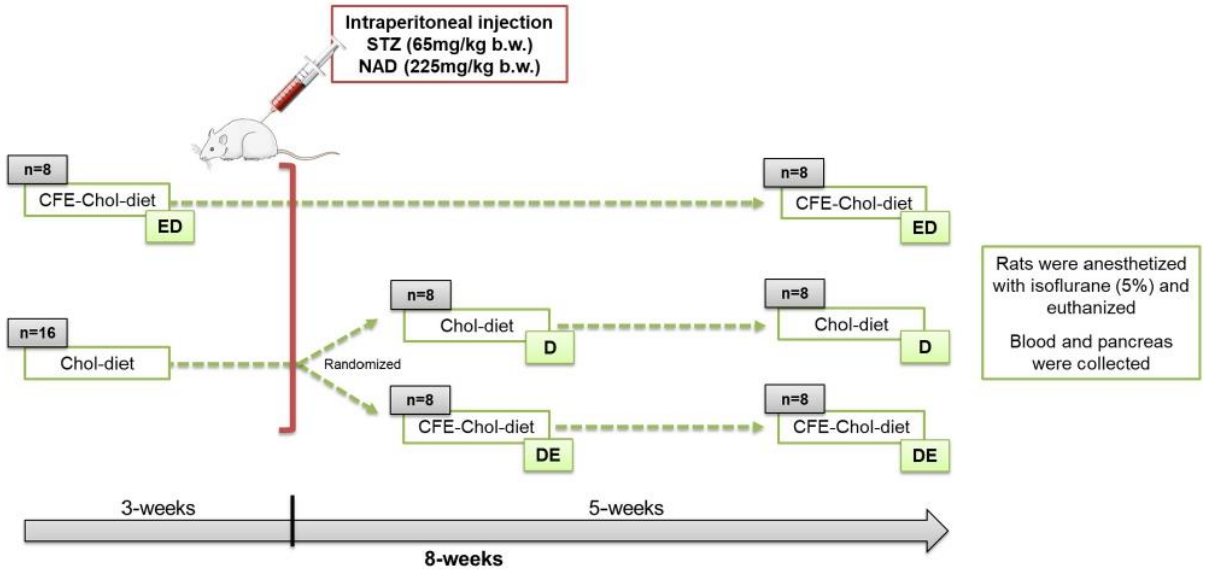

Fig. 1. Experimental design. D, control group; ED, rats fed CFE-Chol-diet since the beginning of the study as preventive strategy; DE, rats fed CFE-Chol-diet since diabetic status as curative treatment. Chol-diet included high saturated fat/high cholesterol and the control-RM; CFE-Chol-diet was high-saturated fat/high cholesterol and the CFE-RM.

at 640 nm in a microplate reader (SPECTROstar Nano). Distilled water and glucose solutions were used as the blank and the standard, respectively. Results were expressed as milligrams of glycogen per gram of tissue.

2.7. Statistical analysis

The results were expressed as mean±S.D. Statistical analysis was performed using SPSS version 25.0 (SPSS Inc., Chicago, IL, USA). Diet effect was tested by analysis of variance (ANOVA) followed by T2-Tamhane or Bonferroni *post hoc* test after assuming inequality or equality of variances, respectively. Hyperglycemic rat distributions among groups were compared by χ^2 test. Differences were considered significant at $P<.05$. Linear correlation analysis was used to explore the relationships between the percentage of immunostained area for LXRα/β and the percentage of steatosis. Correlation coefficient (R) and P value were evaluated to judge the fit of the correlation; $P<.05$ values were considered significant.

3. Results

3.1. Effect of CFE-RM on feed intake, glycemia, insulinemia and HOMA-β

Table 2 shows feed intake, plasma glucose, insulin levels and the HOMA-β index. Extracts did not significantly affect food consumption. Glucose and insulin levels, along with HOMA-β, showed significantly differences between groups (ANOVA at least, $P=.031$). Hyperglycemia (≥300 mg/dl) was found in 7/8 of D rats, 1/8 of ED rats and 0/8 in DE rats (χ^2 test $P<.001$). ED and DE rats displayed significantly lower glycemia compared with D rats (−13% and −23%, respectively; $P<.001$). On the contrary, D rats exhibited the lowest insulin levels, with a fall of 97% ($P<.001$) with respect to ED rats and 69% ($P<.001$) compared with DE ones. Consequently, HOMA-β ($P<.001$) was significantly higher in ED and DE rats than in their D counterparts. Nonsignificant differences were observed between ED *vs.* DE groups ($P>.05$).

3.2. Effect of CFE-RM on pancreatic islet histomorphometry

Considerable degenerative changes of the pancreatic islet of Langerhans were detected in D group (Fig. 2A). Diabetic islets from H&E sections showed irregular outlines and were shrunken with loss of uniform cellular distribution and congested blood capillaries. Some of the remaining β cells had small dense nuclei, vacuolated cytoplasm indicative of degenerative change. In addition, insulin IHQ revealed that D islets had a marked reduction in insulin content, suggesting evidence for insulin degranulation. CFE-RM induced morphological changes in the pancreatic islets (ANOVA $P<.001$) (Fig. 2A). Significant increases in both pancreatic islet number (101.9% and 146.5%; $P=.0037$) (Fig. 2B) and area (162.1% and 125.8%, $P<.001$) (Fig. 2C) were observed in the ED and DE groups with respect to the D group. Pancreatic β cells from CFE-treated groups showed a stronger brown color anti-insulin positive immunostaining, which confirmed the presence of insulin by regeneration of β cells (ED=17.3% and DE=16.7% *vs.* D group; $P=.0112$) (Fig. 2D). Anti-PCNA antibody has been used to determine the potential capacity of β cells for proliferation in both ED and DE islets (Fig. 2E). The number of PCNA-positive cells in islets was increased in ED and DE rats compared with D rats (384% and 534%, respectively; $P<.001$), showing that CFE-RM induced apparent pancreatic islets hypertrophy.

3.3. Effect of CFE-RM on hepatic morphometry and glycogen and lipid contents

Fig. 3 shows the macroscopic and microscopic aspect of the liver as well as body weight, hepatosomatic index, hepatic glycogen and lipid contents. Liver of diabetic rats was enlarged and showed yellowish markings, typical for steatosis (Fig. 3A). In contrast when rats consumed CFE-Chol-diet, liver were smaller and had much better macroscopic aspect (Fig. 3A), showing a lower hepatosomatic index than D group (Fig. 3C). The body weight, hepatic glycogen content (mg/g liver) and the percentages of glycogen area (PAS) and steatosis (H&E) can be found in Fig. 3B, D, E and F, respectively. Both glycogen storage and steatosis were affected by CFE-RM (ANOVA, $P<.001$). ED and DE rats showed higher glycogen accumulation (243% and 236%, $P=.0004$) and a more extensive percentage of PAS glycogen staining area (16.7% and 66.8%, $P=.0011$) than their D counterparts. Histological sections of liver tissue from the three groups showed panlobular steatosis with macrovesicular and microvesicular intracellular lipid droplets, predominantly in portal area (Fig. 3A). However, the percentage of fatty area (steatosis) was significantly lower in ED and DE rats compared with D ones (−17.7% and −10.1%, respectively; $P<.001$). These results suggested that the inclusion of CFE in the RM (CFE-Chol-diet) diet improved the glycogen storage, decreasing steatosis. The comparison between CFE-RM groups (ED and DE) showed significant differences in the percentage of steatosis, lower in ED, and the percentage of PAS-stained area, with DE showing the highest value ($P<.001$). Nonetheless, glycogen content (mg/g) was similar in the two groups ($P>.05$).

3.4. Effect of CFE-RM on the hepatic InsR/PI3K/AKT/GS3K/GLUT2 signaling pathway

Levels and distribution of proteins associated with the hepatic insulin signaling pathway were analyzed by WB and/or IHQ (Fig. 4A–B). Most of the markers implicated in hepatic insulin signaling increased with CFE-RM consumption (ANOVA, $P<.001$). ED and DE rats showed significantly higher levels of InsRβ, PI3K, pAKT2^{ser473} and Glut2 in liver than their D counterparts (Fig. 4A). pGSK3^{ser9} was increased only in DE group with respect to D. The comparison between ED and DE rats revealed differences in PI3K, pAKT2^{ser473} and pGSK3^{ser9} evaluated by IHQ, and Glut2 analyzed by WB. Thus, DE group displayed higher immunostained area for PI3K (28.0%, $P<.05$), pAKT2^{ser473} (32.6%, $P<.001$) and pGSK3^{ser9} (33.75%, $P<.001$) than ED group, while ED showed the highest Glut2 levels (49.7%, $P<.001$). Mostly, the immunoreactivity of hepatic InsRβ, PI3K, pAKT2^{ser473} and pGSK3^{ser9} proteins was located mainly in the cytoplasm of the hepatocytes around portal and centrolobullar areas and also in inflammatory access.

3.5. Effect of CFE-RM on hepatic lipogenic transcription factors

To explore the possible mechanisms of CFE-RM on decreasing liver lipid accumulation, we investigated the expression levels of transcriptionally regulated LXRαβ and its gene target, SREBP-1c and ChREBP genes related to lipogenesis. Fig. 5A–B shows the levels and immunolocalization of hepatic LXRα/β, SREBP-1c and ChREBP transcriptional factors when examined by WB and/or IHQ, respectively. CFE-Chol-diet attenuated the enhanced expression of the three

Table 2
Diet intake, glycemia, serum insulin and HOMA-β in rats fed the three experimental diets

				ANOVA
	ED	D	DE	*P*
Diet intake (g/day)	15.86±0.47	16.48±0.55	16.47±0.77	>.05
Glycemia (mg/dl)	283.7±13.7b	326.0±29.7a	251.0±20.3b	<.001
Serum insulin, μlU/ml	10.59±1.53a	5.41±1.23b	8.99±1.18a	<.001
HOMA-β *	17.28±2.56a	7.24±1.35b	17.27±2.39a	<.001

Values [means±S.D. ($n=8$)] in the same row bearing different lowercase letters were significantly different ($P<.05$; a > b > c; ANOVA followed by T2-Tamhane or Bonferroni *post hoc* test). ED: rats fed the CFE-Chol-diet since the beginning of the study; D: rats fed the Chol-diet; DE: rats fed the CFE-Chol-diet when the diabetic state was confirmed.
* HOMA-β: (20 × fasting serum insulin in μlU/mL)/(fasting glucose in mmol/L)− 3.5; to transform mg/dl into mmol/L of glucose, divide data by 18.0.

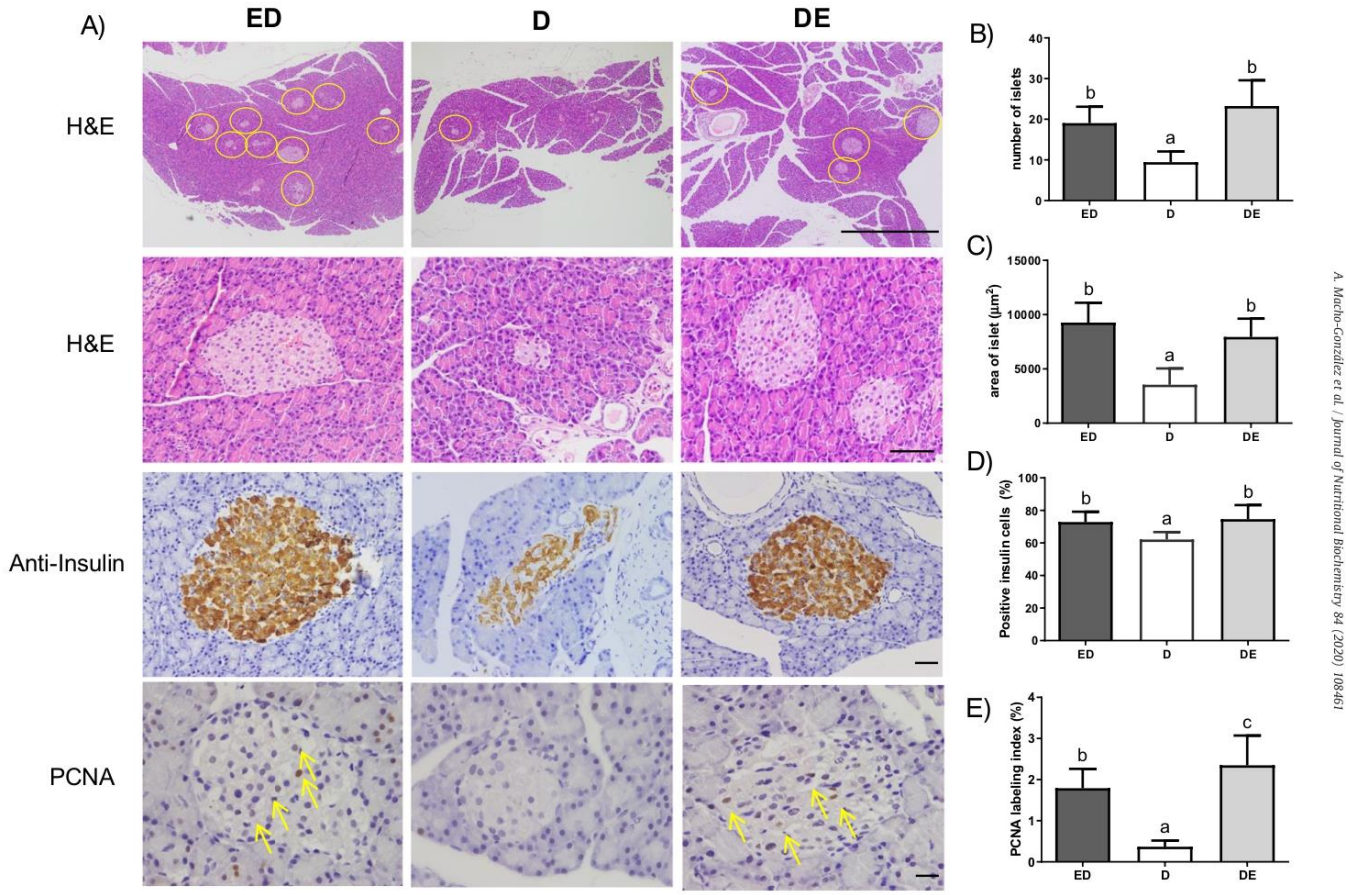

Fig. 2. Pancreatic cell mass is increased in DM rats after CFE consumption. D, control group; ED, rats fed CFE-Chol-diet since the beginning of the study as preventive strategy; DE, rats fed CFE-Chol-diet since diabetic status as curative treatment. (A) Representative images of the rat pancreas showing pancreatic islets. H&E staining (scale bar: 250 µm, magnification 4×; and 50 µm, magnification 20×), and both insulin reactivity (scale bar: 20 µm, magnification 40×) and PCNA staining (scale bar: 20 µm, magnification 40×) are shown in pancreatic tissue by IHQ. (B) Total islet number per pancreatic section. (C) Average islet area (µm^2). (D) Number of β cells per pancreatic islet was determined on insulin-immunostained sections (%). (E) Cell proliferation labeling index (%) was assessed on PCNA immunostained sections. Data represent mean±S.D. ($n = 8$). $P<.05$, different letters indicate significant differences (one-way ANOVA followed by the Tukey posttest).

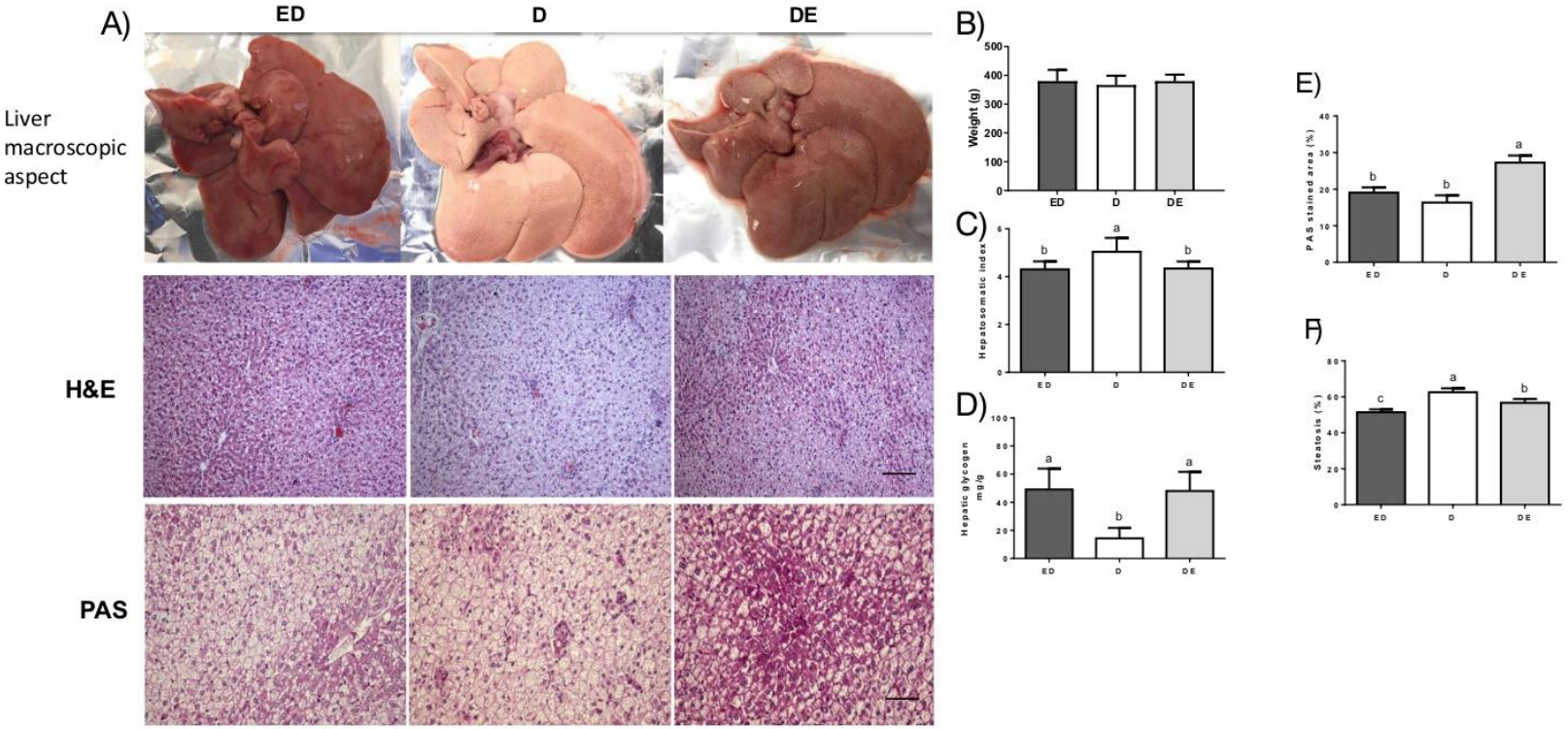

Fig. 3. Macroscopic and microscopic analysis of livers. (A) Representative images of the rat liver from control D group and ED and DE experimental groups. Macroscopic aspect of the liver; H&E and PAS staining (scale bar: 50 μm, magnification 20×). (B) Weight. (C) Hepatosomatic index. (D) Hepatic glycogen (mg/g). (E) PAS staining area. (F) Steatosis. Data represent mean±S.D. ($n = 8$). $P<.05$, different letters indicate significant differences (one-way ANOVA followed by the Tukey posttest). D, control group; ED, rats fed a CFE-Chol-diet since the beginning of the study as preventive strategy; DE, rats fed CFE-Chol-diet since diabetic status as curative treatment.

transcriptional factors induced by T2DM development (ANOVA, $P<.001$). The quantitative analysis demonstrated significantly decreased levels of LXRα/β (ED: IHQ=−22.9%; DE: IHQ=−11.6%), SREBP-1c (ED: IHQ=−21.7%, WB=−36.8%; DE: IHQ=−25.4%, WB=−22.9%) and CHREBP (ED: IHQ=−69.8%, WB=−35.5%; DE: IHQ=−47.7%, WB=−28.7%) compared to D group ($P<.05$ or $P<.01$). Different immunostaining patterns had been observed between D, ED and DE rats (Fig. 5B). D animals presented a homogeneously distributed immunostaining for LXRαβ and SREBP-1c proteins, which was slightly stronger in cytoplasm and nuclei of hepatocytes adjacent to the central vein, as well as in the total of steatotic parenchymal cells. Besides, slices of the liver from D rats also showed strong panlobular CHREBP immunoreactivity, mainly located in both periportal and pericentral hepatocytes but also in lipogranulomas and inflammatory cell infiltrates. The immunolocation of LXRαβ, SREBP-1c and CHREBP in ED and DE rats was mainly located around pericentral area in hepatocytes (Fig. 5B). DE rats displayed a significant rise on the LXRα/β (IHQ=14.5%) and CHREBP levels (IHQ=73.1%, WB=10.5%; $P<.001$) with respect to their ED counterparts. In addition, LXRαβ expression was positively correlated with the amount of intrahepatic fat ($r=0$, $P<.001$) (Fig. 5C) These results seem to indicate that, in both ED and DE groups, there was a reduction of *de novo* lipogenesis (DNL) in comparison with D group.

4. Discussion

In this study, we demonstrated that the consumption of CFE-RM has direct and indirect protective effects on T2DM. In addition, we have investigated the molecular mechanisms of the possible preventive (ED) or curative (DE) antidiabetic effect of CFE-RM. The main findings of this study were the following: (1) The consumption of CFE-RM relieved the pancreatic β-cell dysfunction, which entailed higher levels of insulin and a reduction in hyperglycemia. (2) The improvement in glucidic metabolism by CFE-RM was related to a better InsR/PI3K/AKT signaling pathway, stimulating glucose uptake and glycogen synthesis and increasing insulin sensitivity in liver of diabetic rats. (3) CFE-RM induced a decrease of fatty liver, suppressing DNL activation by down-regulation of transcription factors LXRα/β, SREBP-1 and CHREBP.

The effects of CFE-RM on late-stage T2DM were assessed using a diabetic rat model induced by the conjoined action of a high-saturated-fat/high-cholesterol diet (Chol-diet) together with a low-dose STZ plus NAD injection. This model is close to the typical features of the pathophysiological progression of human T2DM with an insulin secretion decreased by pancreatic β-cell dysfunction [15]. Following previous study of our group, Chol-diet consumption is necessary to establish metabolic alterations and IR, which has been described in Macho-Gonzalez et al. [9], while single injection of low dose of STZ plus NAD causes mild pancreatic β-cell dysfunction without completely compromising the insulin secretion [16,17]. As expected, at the end of the experiment, group D showed clear features of late-stage T2DM, with dysfunctional glucose and lipid metabolisms and relatively low insulin levels. In agreement with other studies [18,19], STZ 60 mg/kg BW selectively damaged pancreatic β cells, which led to the subsequent reduction of insulin secretion, thereby accelerating the hyperglycemic state. Histopathologic examination of the pancreas of D animals revealed that the Langerhans' islets were atrophic, with swollen and small vacuoles in the acinar cells, which explain the decreases in insulin production in D group. As a consequence, a HOMA-β index substantially low supported the failure of pancreatic β-cell function. It is known that T2DM is one of the most common causes of nonalcoholic fatty liver disease (NAFLD) and a more frequent progression to nonalcoholic steatohepatitis (NASH) [20]. The histopathological study of D group liver showed hepatocellular steatosis and accumulation of lipid droplets with low glycogen levels, which are compatible with severe fatty liver degeneration [21]. It ha
described that, in the chronic phase of T2DM, the compr
disposal of glucose as glycogen may further contribute to NA
rerouting excess carbohydrates to fatty acids by DNL pathw
activates SREBP-1c [22]. Our results agree with this hypothes
rats displayed high LXRα/β, SREBP-1c and CHREBP staine
demonstrating the activation of the lipogenesis pathway,
activated by high cholesterol levels present in these animals.

On the contrary, when rats were fed with CFE-RM as pre
strategy (ED) or curative treatment (DE), we detected a delay
T2DM process, giving place to a metabolic stage typical
advanced development of diabetes than the one observed in D
hyperglycemia is coexisting with moderate insulin levels. Thi
can be explained by a lower degeneration of pancreatic
improved insulin secretion sensitivity and glucose disposal,
glycogen levels and reduced intrahepatic lipid content, proba
to the down-regulation of transcriptional factors LXRα/β, SR
and CHREBP. So, the ability of CFE-RM to delay the severity o
can be attributed to different mechanisms discussed below.

First, CFE-RM promoted a considerable protective effect
pancreas, increasing the number of insulin-positive cells in the
Langerhans, showing apparent repopulation and regenerati
cells selectively damaged by Chol-diet-STZ. Consequently,
with CFE-RM led to a marked increase in serum insulin levels in
DE groups with respect to D rats. In addition, partial par
regeneration could be due to increased proliferation of β cells
number of positive PCNA β cells) after the consumption of CFE
is currently accepted that targeting pancreatic β cell is one of th
promising strategies to treat diabetes [23]. Our results mak
with several studies, which reveal that pancreas is one of the ta
dietary polyphenol bioactivity in different diabetic models [24
fact, Lambert et al. [26] have demonstrated that carob-pod-
sweetener was able to enhance insulin synthesis in T2DM indiv
as well as in pancreatic β cells of diabetic obese Zucker rats. A
experiment, Qasem et al. [27] also reported higher HOMA-β
with an increase in the number of β cells after a high dose of c
STZ-NAD-induced T2DM rats. These results suggest that the pro
of pancreatic β cells, stimulating their regeneration, together
subsequent greater insulin release is one of the potential mech
underlying CFE's late-stage antidiabetic effect. With respect
results of ED and DE in the pancreatic parameters, they were
except for PCNA immunostaining, where DE displayed mo
nounced increases. One possible explanation could be the fact
rats were fed with CFE-RM prior to STZ injection, protect
pancreas from injury. On the contrary, when DE rats were tran
to CFE-Chol-diet, islets were already damaged and intense pr
tion of β cells was needed to achieve regeneration. Neverthele
were no differences between insulin levels of the two groups.

Second, the hypoglycemic effect of CFE-RM also involves i
sensitizing mechanisms in the liver. Given our model, liver alte
and IR could be particularly attributed to Chol-diet. Activation
InsR/PI3K/Akt signaling pathway after CFE-RM consumption e
the improvement in insulin sensitivity, leading to a return
endogenous control of glucose homeostasis. At the beginning
pathway, CFE-RM increased at least two-fold the levels of InsR
result is similar to that previously observed with cocoa fla
treatment in human HepG2 treated with high glucose [
expected, CFE-RM also affected insulin signaling downstream
activation of PI3K/Akt pathway, which ended with (a) the inact
of GSK3 that increased glycogen synthesis and (b) the cons
increase in glucose transporter (GLUT2) levels, enhancing
uptake. Previous results of our group have demonstrated th
effective insulin signal transduction after the consumption of C
in an early-stage model of T2DM [29]. In agreement with us, C
et al. [30] noted that flavonoid consumption increased

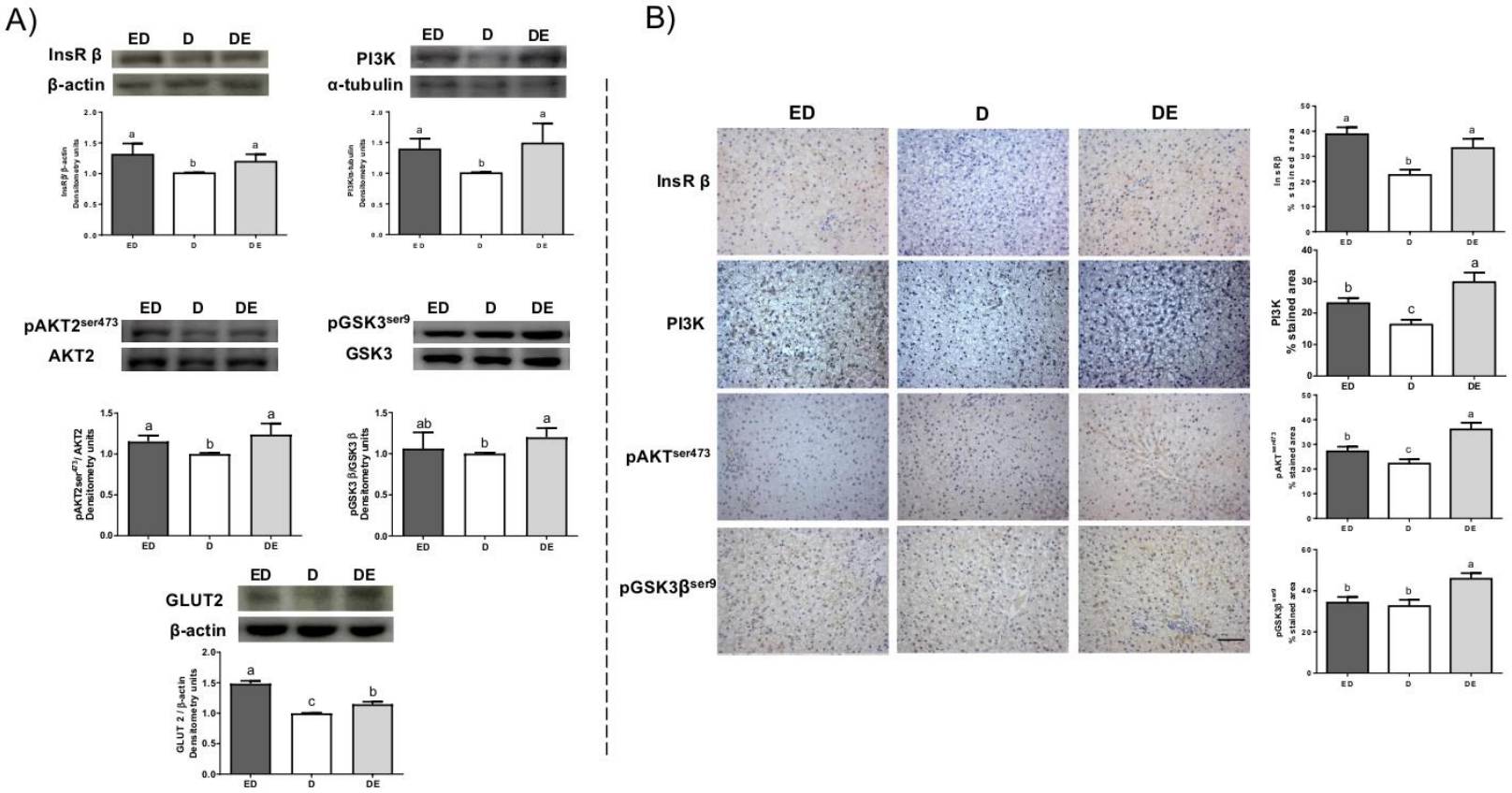

Fig. 4. CFE-enriched meat consumption up-regulates InsR/PI3K/AKT/GSK3/GLUT2 pathway. (A) Representative blot expression levels of InsR-β, PI3K, phosphoAKT2ser473, phosphoGSK3ser9 and GLUT2 proteins over β-actin as loading control by WB analysis; the quantification can be found in the graphs below (densitometry units). (B) Representative microphotographs of liver section immunostained for InsR-β, PI3K, phosphoAKT2ser473 and phosphoGSK3ser9 (scale bar: 50 μm, magnification 20×); the quantification can be found in the graphs beside (% staining area). Data represent mean±S.D. ($n = 8$). $P<.05$, different letters indicate significant differences (one-way ANOVA followed by the Tukey posttest). D, control group; ED, rats fed a CFE-RM since the beginning of the study as preventive strategy; DE, rats fed a CFE-RM since diabetic status as curative treatment.

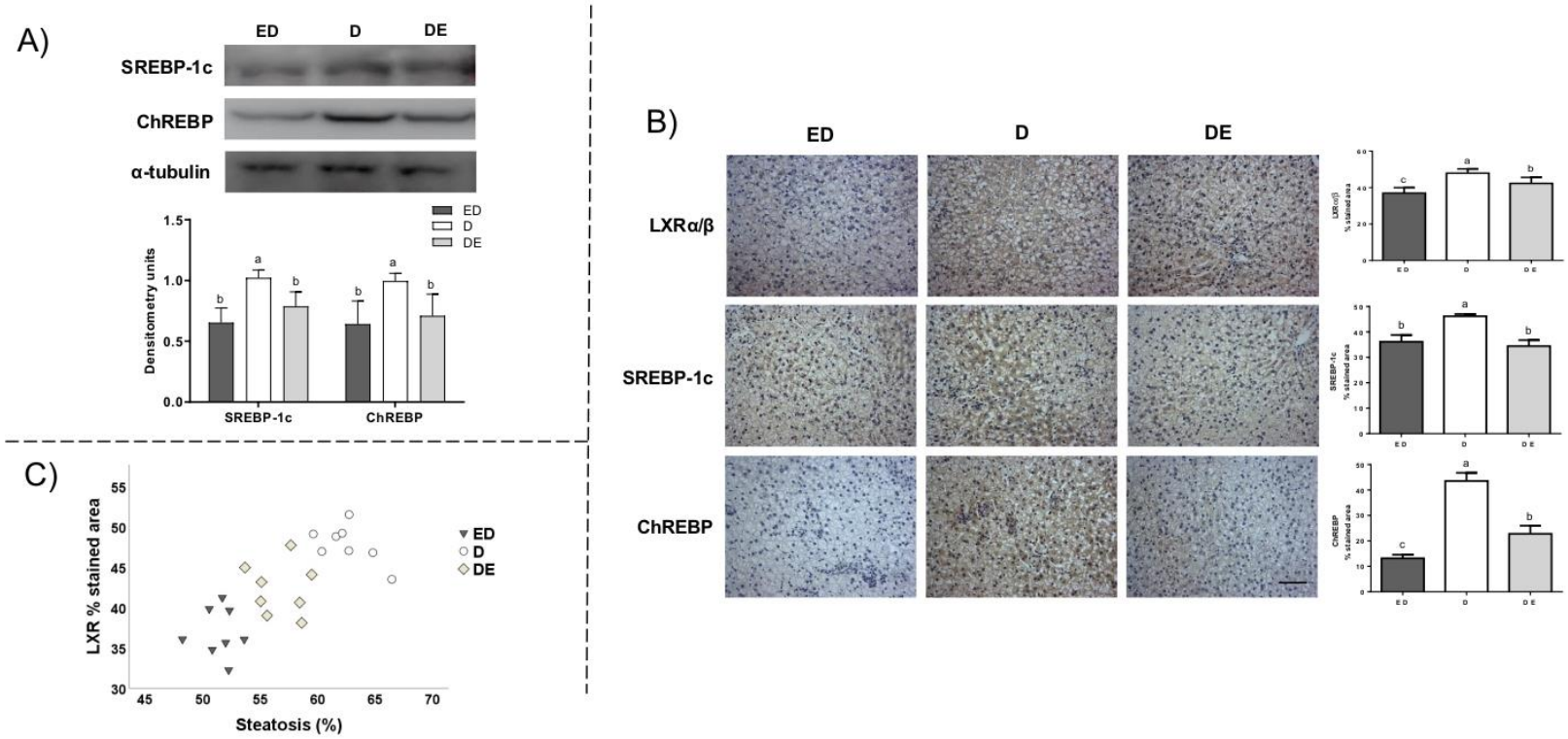

Fig. 5. CFE-enriched meat consumption down-regulates *de novo* lipogenic transcription factors. (A) Representative blot expression levels of SREBP-1c and CHREBP proteins over α-tubulin as loading control by WB analysis; the quantification can be found in the graphs below (densitometry units). (B) Representative microphotographs of liver section immunostained for LXRα/β, SREBP-1c and CHREBP proteins (scale bar: 50 μm, magnification 20×); the quantification can be found in the graphs beside (% staining area). (C) Correlation between LXRαβ stained area and amount of intrahepatic fat. Data represent mean±S.D. ($n = 8$). $P<.05$, different letters indicate significant differences (one-way ANOVA followed by the Tukey posttest). D, control group; ED, rats fed CFE-Chol-diet since the beginning of the study as preventive strategy; DE, rats fed CFE-Chol-diet since diabetic status as curative treatment.

glycogen content by improving PI3K/AKT/GSK3 signaling. Moreover, on GLUT-2, Cordero-Herrera et al. [28] demonstrated that GLUT2 levels and glucose uptake remain normal when HepG2 cells exposed to a high dose of glucose were pretreated with epicatechin or a cocoa polyphenolic extract. Regarding the differences between prevention and treatment, ED rats displayed the highest levels of GLUT-2, while DE showed the greatest increases in PI3K, p-Akt^{ser473} and p-GSK3β^{ser9} protein levels which correlated with PAS staining. However, glycemia and hepatic glycogen were similar in both groups. All together indicate that, in a situation of late-stage T2DM, CFE-RM improved insulin sensitivity, increasing GLUT-2 levels and maintaining glycogen syntheses, which help reduce hyperglycemia.

Finally, concerning hepatic DNL and NAFLD, we found that CFE-RM reduced liver weight and intrahepatic lipid content through down-regulation of transcriptional factors LXRα/β, SREBP-1 and CHREBP, all three closely related to the carbohydrate-mediated regulation of DNL. As previously mentioned, D rats showed an intense immunoreactivity of LXRα/β, although they were euthanized in fasting conditions. This fact, couple with the low levels of insulin found in D animals, suggests that the activation of LXRα/β might be due to oxysterols binding instead to insulin signaling. Another possibility, but it has not been fully proved, is that glucose also participates in LXRα/β regulation. In any case, the levels of SREBP-1c and CHREBP were high in D group, in turn activating the DNL and promoting fat storage in the liver. It has been emphasized that patients with NAFLD present DNL during the fasting state, which can be important in the pathophysiology of this disease [31]. On the other hand, rats fed with CFE-RM showed significantly lower levels of LXRα/β, which are directly linked to the decrease in blood cholesterol [8,9]. In addition, the positive correlation found between LXRα/β and intrahepatic fat deposition suggests that the down-regulation of LXRα/β by CFE-RM may be another important mechanism involved in its antidiabetic effect, which also assumes a protective effect against NAFLD and NASH. In this connection, the down-regulation of the LXRα/β pathway leads to lower hepatic triglyceride synthesis and fat storage through inhibiting SREBP-1c and CHREBP metabolic cascades [32]. Thus, the significant reduction in the levels of SREBP-1c and CHREBP in rats fed with CFE-RM was accompanied by lower hepatic steatosis, suggesting that the simultaneous down expression of this three transcriptional factors in rats fed with CFE-RM induced an important antilipogenic effect. Furthermore, these results make sense with those previously published on the improvement of lipoprotein metabolism in these same animals [9]. Although little is known about the effect of CFE on hepatic lipogenesis, a recent study showed that dietary supplementation with insoluble fiber obtained from carob pod decreased high levels of SREBP-1c liver expression in dyslipidemic rabbits [33]. Concerning the differences between the preventive or therapeutic effect of the consumption of CFE-RM, the ED displayed a stronger down-regulation than the DE of these lipogenic transcription factors accompanied by the lowest fat content in the liver with respect to D and DE rats. Our results reveal beneficial effects of the consumption of CFE-RM in the regulation of the DNL pathway in late-stage T2DM by simultaneously reducing its key regulators: LXRα/β, SREBP-1c and CHREBP, and its importance as a key regulator of liver fat content.

Although this study shows indisputable advantage of CFE-RM consumption in the form of a high-cholesterol/high-saturated-fat diet in late-stage T2DM, the study has some limitations: (a) the study measures and compares metabolic and histological changes just at the end of the study, thus not allowing to evaluate the progression of the disease that would demand a rather large number of rats. Present ethical rules prescribe to use the lowest number of animals possible in research, thus limiting the study possibilities. (b) The study was performed only in male and relatively young rats. (c) Only a CFE dose was tested, demanding more studies in a short future.

In summary, the preventive or therapeutic consumption of CFE-RM enhances insulin sensitivity by directly boosting the regeneration of pancreatic β cells, which indirectly improves the control of hepatic glucose uptake and glycogen storage through activation of InsR/PI3K/AKT/GSK3/GLUT2 pathway. In addition, CFE-RM reduced the accumulation of liver lipids and DNL by down-regulating the levels of LXRα/β, SREBP-1c and ChREBP, inducing an important antilipogenic effect. This study elucidates for the first time the molecular mechanisms responsible, at least in part, for the antidiabetic effect of the CFE-RM. These promising results support that (a) the inclusion of CFE in a meat matrix is a good strategy to obtain functional meat and (b) the dietary consumption of CFE-RM seems to be an effective and complementary tool to prevent or manage late-stage T2DM.

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Declaration of interest

The author declare that they have no competing interests.

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Publicación 10

Carob fruit extract-enriched meat, as preventive and curative treatments, improves gut microbiota and colonic barrier integrity in a late-stage T2DM model

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Antecedentes: La dieta es un factor clave en el mantenimiento de la homeostasis entre la microbiota intestinal y el huésped, cuya alteración puede conducir a la progresión de diversas enfermedades como la DMT2 (Sharma & Tripathi, 2019). La fibra y los polifenoles se han postulado como unos prometedores agentes prebióticos, capaces de promover el crecimiento de especies bacterianas beneficiosas y mejorar la salud intestinal (Anhe y cols., 2015; Scott y cols., 2013). El CFE es un compuesto rico fibra insoluble y PACs, el cual ha demostrado efectos hipoglucemiantes e hipolipemiantes (Artículos 1 y 2), a la vez que reduce la disfunción pancreática y mejora la sensibilidad a la insulina hepática (Artículos 7 y 9). Sin embargo, hasta la fecha no se ha descrito su efecto sobre la microbiota intestinal y la integridad de la barrera colónica.

Hipótesis: El consumo de un cárnico enriquecido en CFE mejora la microbiota intestinal y reduce las alteraciones de la integridad de la barrera colónica en un modelo de estadio tardío de DMT2.

Resultados: El estudio histopatológico del colon mostró una mucosa colónica distal alterada en el grupo D, con bajos niveles de células caliciformes y una distribución anormal de las uniones estrechas (ocludina y zonulina-1). La inclusión del cárnico enriquecido en CFE como estrategia preventiva (ED) presentó niveles más altos de especies bacterianas beneficiosas (*Bacteroides* spp., *Bifidobacterium* spp., *F. prausnitzii* y *Lactobacillus* spp.), AGCC (acetato, propionato y butirato) y una mejor integridad de la mucosa colónica distal (mayor profuncidad de criptas, células caliciformes, recambio celular y uniones estrechas) en comparación con el grupo control D. En cambio, el consumo del cárnico enriquecido en CFE como tratamiento terapéutico (DE) reveló menor abundancia de especies bacterianas potencialmente dañinas (*Enterobacteriaceae* y *Enterococcus* spp.) y de zonulina-1 en la mucosa colónica distal y mayores niveles de ocludina en ambos segmentos colónicos.

Conclusiones: El consumo del cárnico enriquecido en CFE como estrategia preventiva bloqueó los efectos dañinos de la dieta alta en grasas saturadas y colesterol sobre la microbiota intestinal, la producción de AGCC y la pérdida de la integridad de la barrera colónica. En contraste, el consumo del cárnico enriquecido en CFE como tratamiento terapéutico no logró los efectos esperados, mostrando una acción local sobre las uniones estrechas, en un intento de reforzar la barrera colónica. La utilización de CFE como ingrediente funcional parece una adecuada estrategia nutricional para incrementar el aporte de fibra y PACs, además de para minimizar el impacto de las dietas occidentales sobre la fisiopatología de la DMT2.

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Carob fruit extract-enriched meat, as preventive and curative treatments, improves gut microbiota and colonic barrier integrity in a late-stage T2DM model

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ABSTRACT

Epidemiological and experimental studies have suggested that dietary fiber and proanthocyanidins play an important role on gut microbiota (GM), colonic integrity and body health. Type 2 Diabetes Mellitus (T2DM) is a prevalent disease in which the modifications in the GM and colonic markers stand out. This manuscript hypothesizes the consumption of functional meat enriched in carob fruit extract [CFE; CFE-restructured meat (RM)] ameliorates the dysbiosis and colonic barrier integrity loss in a late-stage T2DM rat model induced by the conjoint action of a high-saturated-fat/high-cholesterol diet (Chol-diet) and a low dose of streptozotocin (STZ) plus a nicotinamide (NAD) injection. Three groups of eight rats were used: (1) D group, a T2DM control group, fed the Chol-diet; (2) ED group, a T2DM preventive strategy group fed the CFE-Chol-diet since the beginning of the study; and (3) DE group, a T2DM curative treatment group, fed the CFE-Chol-diet once the diabetic state was confirmed. The study lasted 8 weeks. Amount and variety of GM, feces short-chain-fatty acids (SCFAs), colonic morphology [crypt depth and density, goblet cells, proliferating cell nuclear antigen (PCNA) and transferase dUTP nick end labelling (TUNEL) indexes] and tight junctions were evaluated. A global colonic index combining 17 markers (GC_{index}) was calculated. ED rats displayed higher levels of GM richness, SCFAs production, crypt depth, and goblet cells than the D group. DE group showed lower *Enterobacteriaceae* abundance and greater TUNEL index and occludin expression in the distal colon than D counterpart. GC_{index} differentiated the colonic health status of the experimental groups in the order (ED > DE > D; $P < 0.001$) as a 17–51 range-quotation, ED, DE, and D groups displayed the values 43, 32.5, and 27, respectively. Thus, CFE-RM used as a T2DM preventive therapy could induce higher GM richness, more adequate SCFAs production, and better colonic barrier integrity. Furthermore, CFE-RM used with curative purposes induced more modest changes and mainly at the distal colonic mucosa. Further studies are needed to confirm this study's results, to ascertain the benefits of consuming proanthocyanidins-rich fiber during different T2DM stages.

Abbreviations: CFE, carob fruit extract; CFE-RM, CFE-enriched meat; CFU, colony-forming units; Chol-diet, high-saturated-fat/high-cholesterol diet; CM_{index}, colon morphology index; GC_{index}, global colonic index; GM, gut microbiota; GM_{index}, gut microbiota index; H&E, hematoxylin and eosin; ITR, intertertile range; NAD, nicotinamide; PAS, periodic Acid-Schiff; PACs, proanthocyanidins; PCNA, proliferating cell nuclear antigen; RM, restructured meat; ROC, receiver operating characteristic; SCFAs, short-chain fatty acids; $SCFAs_{index}$, short-chain fatty acids index; STZ, streptozotocin; T2DM, Type 2 Diabetes Mellitus; TJ, tight junctions; TJ_{index}, tight junctions index; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; ZO-1, zonula occludens-1.

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1. Introduction

Diet is a key factor in maintaining homeostasis between the gut microbiota (GM) and the host, by regulating its composition and metabolism (Zmora, Suez, & Elinav, 2019). Western countries, with diets rich in animal proteins, fats, and cholesterol, and low in dietary fiber, present an increased risk of developing pathologies, such as Type 2 Diabetes Mellitus (T2DM) (Muñoz-Garach, Díaz-Perdigones, & Tinahones, 2016; Sircana et al., 2018). However, the scientific community has not determined whether GM dysbiosis and loss of barrier integrity are the cause or consequence of this disease (Gurung et al., 2020; Sircana et al., 2018).

T2DM physiopathology implies deleterious effects on intestinal health because it has been demonstrated that it affects the colonic structure and function inducing gastrointestinal symptoms such as abdominal pain, bloating, diarrhea, and constipation (Sharma & Tripathi, 2019). Likewise, animal and human studies have reported that diabetes induced by high-fat diets and dietary cholesterol modifies GM toward a pathological phenotype, decreasing bacterial amount and diversity, causing dysbiosis with a predominance of less beneficial populations (Cani et al., 2007; Hildebrandt et al., 2009; Hu et al., 2015; Murphy, Velazquez, & Herbert, 2015). Moreover, chronically elevated glycemia, a characteristic of late-stage T2DM, appears to be a direct cause of GM alteration and intestinal mucosal barrier loss by promoting an alteration of tight junctions (TJs) and contributing to microbial adhesion and translocation (Thaiss et al., 2018).

T2DM animal models have been extensively used to assess molecular mechanisms involved in this disease (Premilovac et al., 2017; Sharma, Ashhar, Aeri, & Katare, 2019; Skovsø, 2014). Our research group has reported that rats fed a high-saturated-high-cholesterol diet (Chol-diet) receiving a streptozotocin plus nicotinamide (STZ-NAD) injection developed a late-stage T2DM based on observed high glycemia, normal-low insulinemia, and β-cell pancreas value decreases (Macho-González, López-Oliva, et al., 2020). Even though pharmacotherapy is essential for late-stage T2DM patients, lifestyle interventions, including dietary and physical activity changes, remain the treatment cornerstone (Kahn, Cooper, & Del Prato, 2014; Pozzilli & Fallucca, 2014). Including foods rich in fiber and polyphenols can exert healthy properties and modulate GM richness and diversity (Lazar et al., 2019; Saldaña, Bastida, Macho-González, & Sánchez-Muniz, 2020). Besides their effects on GM, fiber consumption has also shown improved intestinal permeability, suppressed inflammation; by reducing the endotoxemia level, and regulates carbohydrate and lipid metabolisms during T2DM (Xiao et al., 2014). Thus, fiber has been suggested to be an adequate nutritional alternative for T2DM patients. Carob fruit extract (CFE) is a product obtained from the carob fruit pulp rich in dietary fiber and proanthocyanidins (PACs), non-digestible flavonoids that reach the colon and are metabolized by the GM, exerting many health properties (Goulas, Stylos, Chatziathanasiadou, Mavromoustakos, & Tzakos, 2016; Macho-González, Garcimartín, et al., 2020; Rtibi et al., 2017). It has been reported that carob fruit has multiple pharmacological activities in the digestive tract including antioxidant, antidiarrheal, antibacterial, anti-ulcer, and anti-inflammatory actions (Rtibi et al., 2017). Besides, PACs from other sources similar to those containing CFE, promoted beneficial bacteria population growth, increased enteroendocrine hormone secretion, and modulated GM and short-chain fatty acids (SCFAs) production in high-fat diet fed rats (Casanova-Marti et al., 2018; Lee et al., 2018).

In this study, we consider the potential CFE effect on GM and colonic barrier integrity. Thus, given the difficulty of modifying population dietary habits, different nutritional strategies are emerging to overcome this barrier, such as the development of functional foods. We recently demonstrated that CFE as a functional ingredient of restructured meat (RM), improved pancreatic β-cell dysfunction, hepatic insulin signaling and lipogenesis in a late-stage T2DM model (Macho-González et al., 2019; Macho-González,López-Oliva, et al., 2020). However, the effect of CFE-enriched meat (CFE-RM) consumption on GM has not been evaluated. Therefore, we hypothesized that CFE-RM improves GM and alleviates diabetes-induced alterations of the colonic mucosa integrity in T2DM rat models. The objective of this study was to evaluate the protective effect of CFE-RM consumption as a preventive or curative treatment on (a) GM dysbiosis, (b) SCFAs production, and (c) the basic structure and integrity of the colonic mucosa in a late-stage T2DM model induced by STZ-NAD injection in the frame of a Chol-diet.

2. Materials and methods

2.1. Carob fruit extract restructured meat and diet preparation

CFE is a natural insoluble dietary fiber with high content in PACs obtained from the carob pulp following the procedure described in WO2004/014150 patent (Ruiz-Roso, Requejo, Pérez-Olleros, Martín-Casero, & Haber, 2004) and whose composition has been described by Macho-González et al. (Macho-González et al., 2018). Briefly, CFE extract's average composition is proteins, 4.5–7%; fats, 0.5–1%; sugars, 1.5–3.5%; ashes, 3–4% and total dietary fiber (rich in condensed and non-condensed tannins in which flavan-3-ol units), 74–84%.

Details of restructured meat (RM) formulation and diet preparation have been previously published (Macho-González et al., 2019). Briefly, RM was prepared using lean minced meat (50% pork:50% veal) as described by Schultz-Moreira et al. (2010). CFE-RM was homogeneously prepared mixing CFE (4 g/kg restructured meat) with lean mixed meat. The resulting RMs were freeze-dried and ground in a chilled meat cutter (Stephan Universal Machine UM5; Stephan, Söhne GmbH and Co, Hameln, Germany). For each kilogram of diet, 30% of RM, 5% of cellulose powder and 65% of a purified diet formulation (reference U8959, version 180; Panlab S.L., Barcelona, Spain) were mixed and sieved three times until a homogenous powder was obtained.

Diets were designed to contain 50% energy from fat (20.4% saturated fat), 36% energy from carbohydrate, and 14% energy from protein. Two experimental semisynthetic diets were prepared: (a) Diet containing the control-RM and 1.4% cholesterol and 0.2% cholic acid (98% purity) (Chol-diet); and (b) identical to the Chol-diet but containing 1.14% CFE [CFE-RM (CFE-Chol-diet)]. The CFE-Chol-diet contained 1.14% less dietary cellulose to keep similar total amount of fiber in diets (Table 1). CFE-RM provided 125 mg/kg b.w. of CFE (Macho-González et al., 2019) the amount proved to induce very positive effects on lipemia and blood glucose (Macho-González et al., 2017; Macho-González et al., 2018). Larger CFE amounts (e.g. 500 mg or 1000 mg/kg b.w.) would not be technologically feasible in the meat industry and would represent a high to very high dose when extrapolating them for humans.

2.2. Experimental design

Twenty-four male Wistar rats aged two-months old were obtained from Harlan Laboratories models (Harlan S.L., Barcelona, Spain) and housed in pairs under controlled temperature and light (22.3 ± 1.9 °C and 12-h light/dark cycle, respectively) at the Centro de Experimentación Animal of the University of Alcalá [Madrid, Spain (register no. ES280050001165)]. Tap water and food were provided ad libitum. By the 3rd week of the study, T2DM was induced by intraperitoneal injection of STZ (65 mg/kg b.w.) and NAD (225 mg/kg b.w.) (Sigma-Aldrich, Madrid, Spain).

The rats were divided into three groups of eight animals: (1) D group, a T2DM control group, fed the Chol-diet; (2) ED group, a T2DM preventative strategy group, fed the CFE-Chol-diet since the beginning of the study; and (3) DE group, a T2DM curative treatment group, fed the CFE-Chol-diet once the diabetic state was confirmed. The study lasted 8 weeks. The experimental design is summarized in the Supplemental Fig. S1. Food consumption and body weight was tested daily as previously reported (Macho-González et al., 2019). Feces, to determine total fecal deposition and composition, were collected and weighed every day

Table 1
Composition of the experimental diets fed to diabetic rats.

Dietary Components	Chol-Diet	CFE-Diet
Protein, %	14.0	14.0
Fat, %	49.0	49.0
Cholesterol, g/kg	9.83	9.83
SFA:MUFA:PUFA ratio	2.10/2.32/1	2.10/2.32/1
Energy content*, MJ/kg	20.36	20.36
Ingredients, g/kg		
Sucrose	68.25	68.25
Corn starch	275.73	275.73
Casein	94.25	94.25
Maltodextrin	94.25	94.25
Cellulose	48.86	48.86
PM 205B SAFE	50.05	50.05
PV 200 SAFE	7.15	7.15
Soybean oil	47.91	47.91
L-Cysteine	2.02	2.02
Cholesterol	9.1	9.1
Cholic acid	1.3	1.3
Freeze-dried restructured meat	301.14#	301.14#

Chol-diet, diet containing the control-RM and 1.4% cholesterol and 0.2% cholic acid; CFE-diet, diet containing the carob fruit extract-RM (CFE-RM) and 1.4% cholesterol and 0.2% cholic acid. *Data were calculated according to the energy equivalents for carbohydrate [16.73 kJ/g (4.0 kcal/g)], fat [37.65 kJ/g (9.0 kcal/g)], and protein [16.73 kJ/g (4.0 kcal/g)]. SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; PM 205B SAFE, Mineral mix; PV 200 SAFE, Vitamin Mix. #Control-RM was formulated with cellulose (4 g/kg) while CFE-RM with carob fruit extract (4 g/kg), in order to maintain the same dietary fiber amount contribution.

during the final week. To avoid inter-assay variations, overnight fasted rats were taken, one from each group at a time, anesthetized with isoflurane (5% vol/vol) and euthanized. Blood was collected in cold tubes from the descending aorta with a heparinized syringe and placed in ice until processing. Feces were carefully removed from the final colon. Afterwards, the whole colon was dissected and longitudinally measured from the end of the cecum to the rectum and weighed without feces. Pieces 0.5 cm long from the proximal and distal segments were resected. The caecum was weighed with the fecal content inside.

All experiments were performed in compliance with Directive 2010/63/EU of 22 September 2010 (amended by Regulation (EU) 2019/1010) on the protection of animals used for scientific purposes. The study was approved by the Spanish Science and Technology Advisory Committee (project AGL2014-53207-C2-2-R) and by the Ethics Committee of the Universidad Complutense de Madrid (Spain).

2.3. Gut microbiota analysis

Fecal samples directly removed from the distal colon were collected in sterile tubes and immediately frozen at −80 °C until analyses. Bacterial DNA was extracted from 180 to 220 mg of each fecal sample using an optimized protocol including bead beating with the FastPrep instrument (MP Biomedicals, California, USA) and DNA extraction with commercial QIAamp DNA Stool Mini Kit columns (Qiagen N.V., Venlo, Netherlands) following the manufacturer instructions. Details of the protocol have been previously described Redondo et al. (2019). The extracted DNA was kept frozen at −80 °C until further use. DNA concentration was measured in a Nanodrop ND-1000spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

SYBR-Green real-time PCR (qPCR) was performed for the detection of 16S rRNA genes with specific primers (Table 2) targeted to these bacterial groups: *Bacteroides*, *Blautia coccoides–Eubacterium rectale* group, *Clostridium leptum* group, *Faecalibacterium prausnitzii*, *Bifidobacterium* spp., *Lactobacillus* spp., *Enterobacteriaceae*, and *Enterococcus* spp. qPCR experiments were conducted with an AriaMix equipment (Agilent Technologies, Palo Alto, CA, USA) using SYBR Green QPCR Master Mix (Agilent Technologies) in reaction mixtures and following amplification programs previously described (Redondo et al., 2019). A standard curve for each qPCR assay was used for the quantification of target bacterial DNA.

2.4. Colon morphology and goblet cell analysis

The colon sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, dehydrated, and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) and/or periodic Acid-Schiff (PAS) reagent according to the routine methods. Images of the sections were captured at ×200 magnification using a Leica DM LB2 microscope equipped with Leica DFC 320 digital camera (Leica Microsystems, L'Hospitalet del Llobregat, Spain) and a morphometric analysis were conducted using Fiji ImageJ v1.52j software software (National Institute of Health, USA). The crypt depth was measured from H&E slices and was determined as the number of cells per hemi-crypt. The tissue expression level of the neutral mucin glycoprotein was determined with PAS staining and calculated as the number of PAS positive cells per crypt. Only crypts extended from the base to the brush border of the luminal surface were considered. Data are presented as the average number of total cells per crypt (crypt-depth) and of stained goblet cells per crypt.

Table 2
Primer sets used in the quantitation of 16S rRNA genes by qPCR.

Target organism	Primer set	Sequence (5 to 3)	Product size (bp)	Annealing temp (°C)	Reference
Bacteroides spp.	Bfra-F Bfra-R	ATAGCCTTTCGAAAGRAAGAT CCAGTATCAACTGCAATTTTA	495	50 °C	(Matsuki, Watanabe, Fujimoto, Takada, & Tanaka, 2004)
Blautia coccoides -Eubacterium rectale group	gCcoc-F gCcoc-R	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	440	50 °C	(Matsuki et al., 2004)
Clostridium leptum group	sg-Clept-F sg-Clept-R3	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	239	50 °C	(Matsuki et al., 2004)
Bifidobacterium spp.	Lm26-F Bif228-R	GATTCTGGCTCAGGATGAACG CTGATAGGACGCGACCCCAT	211	60 °C	(Walter et al., 2001)
Faecalibacterium prausnitzii	Fprau223F Fprau420R	GATGGCCTCGCGTCCGATTAG CCGAAGACCTTCTTCCTCC	199	58 °C	(Bartosch, Fite, Macfarlane, & McMurdo, 2004)
Lactobacillus spp.	LbF LbR	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	200	56 °C	(Heilig et al., 2002)
Enterobacteriaceae	Eco1457F Eco1652R	CATTGACGTTACCCGCAGAAGAAGC CTCTACGAGACTCAAGCTTGC	195	63 °C	(Bartosch et al., 2004)
Enterococcus spp.	Enteroc_F Enteroc_R	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	123	61 °C	(Rinttilä, Kassinen, Malinen, Krogius, & Palva, 2004)

2.5. Immunohistochemistry

Colonic tissue sections were deparaffinized, the citrate antigen recovered, and the endogenous peroxidase quenched with 3% hydrogen peroxide. Then, sections were incubated overnight at 4 °C with the following primary polyclonal antibodies: anti-proliferating cell nuclear antigen (PCNA), anti-occludin and anti-zonula occludens-1 (anti-ZO-1) (Santa Cruz Biotechnology Quimigen, Madrid, Spain). After washes, sections were covered with the appropriated biotinylated secondary antibody. Immunochemical staining was performed by using streptavidin-biotin conjugated horseradish peroxidase (Sigma Aldrich, Madrid, Spain) and visualized by incubation with 3, 3′-diaminobenzidine (DAB) (Sigma Aldrich). Sections were counterstained using Harris's hematoxylin, dehydrated and mounted. Fifteen fields per section per rat (×400 magnifications for image analysis) were selected and quantified. The percentage of positive DAB area was assessed by individually selecting each colonic crypt and applying the colour deconvolution plugin from Fiji ImageJ v1.52j to ZO-1 and occludin. ZO-1 and occludin immunostaining were expressed as the cell number (%) stained per crypt.

For PCNA labelling index quantification, at least 20 perpendicular well-oriented crypts were examined in each animal under light microscopy at ×400 magnification. Labelling index (%) was calculated as the number of positive nuclei × 100/total number of cells/crypt column height.

2.6. Assessment of apoptosis

The terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was used to determine apoptotic colonocytes of proximal and distal sections according to the López-Oliva et al. method (López-Oliva, Pozuelo, Rotger, Muñoz-Martínez, & Goñi, 2013).

The TUNEL labelling index (%) was calculated as the number of apoptotic cells × 100/total number of cells/crypt column height. For the quantification of the TUNEL labelling index at least 50 perpendicular well-oriented crypts were examined and counted for each animal at ×400 magnification using a Leica DM LB2 microscope equipped with a Leica DFC 320 digital camera (Leica Microsystems).

2.7. Feces SCFAs levels

Feces (0.1 g) directly removed from distal rat colons after euthanization were weighed and suspended in 1 mL of water with 0.5% phosphoric acid and frozen at −20 °C immediately after collection. SCFAs levels were determined in feces according to the method described by Álvarez-Cilleros et al. (2020). Briefly, feces were homogenized with a vortex for 2 min and centrifuged at 17,949*g* for 10 min. The analytes (2 μL) were injected in the splitless mode into an Agilent 7890A gas chromatography system (Agilent Technologies) equipped with a 5975C mass spectrometer detector and an Agilent DB-WAXtr column (100% polyethylene glycol, 60 m, 0.325 mm, 0.250 μm). Helium was used as carrier gas at 1.5 mL/min. The mass spectrometer was tuned during measurements single-ion monitoring mode was used for signal quantification. The ionization source and quadrupole temperatures were 230 °C and 150 °C, respectively. The electron-impact ionization energy was 70 eV. A stock solution containing a mixture of standards (WSFA-2; Sigma-Aldrich) was treated as samples and diluted to obtain a calibration curve ranging from 2 to 10.000 μM to which an internal standard (4-methylvaleric acid) was added. Concentrations of SCFAs were expressed as μM/g of feces.

2.8. Colonic markers concurrence

Tertile values for each colonic variable were calculated in the 24 rats studied. Differences in variable distribution among groups were later tested. Values for the branched fatty acids (isovaleric and isobutyric) and *Enterobacteriaceae* at the first, second, and third tertiles contribute with the values 3, 2, and 1, respectively; whereas the rest of variables at the first, second, and third tertiles contribute with the values of 1, 2, and 3, respectively.

Later some indexes were obtained considering physiological and or pathological similarities and associations between variables that would permit us to obtain one or some single indexes with higher discriminant power than a single marker (Gesteiro, Bastida, & Sánchez-Muniz, 2013; Ruperto, Barril, & Sánchez-Muniz, 2017). The average score was calculated considering the values 1, 2 or 3 for the 1st, 2nd or 3rd tertiles, respectively, and the number of rats into each tertile; thus, an average value close to three means that most of the animals were located into the 3rd tertile. Thus, a GM index (*Bifidobacterium* spp., *F. prausnitzii*, *Lactobacillus* spp. and *Enterobacteriaceae*) was obtained considering the association of four species and their value calculated by adding the tertile value of each parameter (GM_{index} range, 4 to 12); total $SCFA_{index}$ [ramified plus non-ramified fatty acids ($SCFA_{index}$ range, 6 to 18)]; the colon morphology index CM_{index} (crypt depth, crypt density, positive goblet cells/crypt, PCNA index, and TUNEL index) (CM_{index} range, 5 to 15); the TJ_{index} was calculated adding tertile values of occludin plus ZO-1 (TJ_{index} score range, 2 to 6). Last, the global colonic index (GC_{index}), considering a conjoint marker of 17 variables, was obtained from adding of tertile values of GM_{index} + $SCFA_{index}$ + CM_{index} + TJ_{index} (GC_{index} range from 17 to 51).

2.9. Statistical analysis

The results were expressed as mean ± SD or median (intertertile range, ITR). Differences among groups were assessed by one-way ANOVA followed by a T2-Tamhane or Bonferroni *post-hoc* test, after assuming inequality or equality of variances, respectively; Kruskal-Wallis test followed by a non-parametric multiple comparison *post hoc* test; or chi-squared test. Proximal and distal colon results were compared using the unpaired Student's *t*-test. Contingency tables were used to determine differences of rat distribution from the three experimental groups within the three tertiles of the different indexes assayed. The Pearson product-moment correlations between GM, fasting parameters, SCFAs, and colonic morphology were determined. Differences were considered significant at $P < 0.05$. Statistical analysis was performed using SPSS version 25.0 (SPSS Inc., Chicago, Illinois, USA) and graphs were drawn with GraphPad Prism version 8 (GraphPad software, Inc., La Jolla, California).

3. Results

3.1. Fasting plasma determinations, colon characteristics, and fecal excretion

Fasting glycemia, insulinemia, triglyceridemia, and cholesterolemia results have been reported elsewhere (Macho-González et al., 2019; Macho-González,López-Oliva, et al., 2020). Briefly, all fasting parameters were significantly affected by CFE-Chol-diet. ED followed by DE rats showed higher insulinemia, lower glycemia and cholesterolemia, higher islet size and beta-cell count than their D counterparts (Supplemental Fig. S2) (Macho-González,López-Oliva, et al., 2020).

Table 3 summarizes the colon characteristics and fecal excretion results from the different groups. Fecal excretion and moisture were significantly affected by diet (ANOVA at least $P = 0.007$). Further, ED and DE rats showed higher fecal excretion than D counterparts (23.5% and 18.7%, respectively; at least $P < 0.05$). Feces of ED and DE groups presented higher moisture content compared to the D group (32.2% and 18.4%; both, $P < 0.05$).

3.2. Effect of CFE-RM on gut microbiota

Table 4 shows the PCR quantification of 16S rRNA genes of the main

Table 3
Colon characteristics and fecal excretion of the three experimental groups.

	ED	D	DE	ANOVA P
Colon length (cm)	15.38 ± 1.16	15.31 ± 1.91	15.88 ± 1.55	NS
Colon weigth (g)	1.53 ± 0.41	1.41 ± 0.32	1.81 ± 0.65	NS
Caecum weight (g)	2.54 ± 0.46	2.49 ± 0.48	2.89 ± 0.69	NS
Feces colon weight (g)*	0.91 ± 0.28	0.64 ± 0.21	0.78 ± 0.37	NS
Fecal excretion (g/day) **||	2.31 ± 0.20a	1.87 ± 0.03b	2.22 ± 0.36a	0.007
Fecal moisture (%)||	18.56 ± 1.55a	14.04 ± 1.66b	16.62 ± 1.40a	<0.001

Labeled means in a row bearing different letters were significantly different (at least $P < 0.05$; a > b > c; one way ANOVA followed by the Bonferroni or T2 Tamhane *post hoc* test). ED: rats fed the CFE-diet since the beginning of the study; D: rats fed the Chol-diet; DE: rats fed the CFE-diet when the diabetic state was confirmed. *Feces collected directly from inside the colon at the sacrifice time; **Data are wet matter weights. Feces daily collected during the last experimental week from rat cages. NS, non-significant ($P > 0.05$)

Table 4
PCR quantitation of 16S rRNA genes of the main bacterial groups in colonic feces of the three experimental groups.

	ED	D	DE	ANOVA P
Bacteroides spp.	10.08 ± 0.41a	9.24 ± 0.32b	9.60 ± 0.40ab	0.001
Blautia coccoides -Eubacterium rectale group	11.42 ± 0.33b	11.96 ± 0.25a	11.33 ± 0.50b	0.005
Clostridium leptum group	7.86 ± 0.37b	8.24 ± 0.12a	8.13 ± 0.19ab	0.017
Bifidobacterium spp.	7.04 ± 0.39a	6.25 ± 0.33b	5.96 ± 0.37b	<0.001
Faecalibacterium prausnitzii	8.61 ± 0.26a	8.22 ± 0.25b	8.27 ± 0.25ab	0.012
Lactobacillus spp.	8.69 ± 0.18a	8.24 ± 0.26b	7.69 ± 1.03ab	0.015
Enterobacteriaceae	6.85 ± 0.57a	7.16 ± 0.74a	5.96 ± 0.67b	0.004
Enterococcus spp.	5.92 ± 0.52ab	6.36 ± 0.72a	5.36 ± 0.41b	0.008

Mean (Lg CFU/g. feces ± SD). Labeled means in a row bearing different letters were significantly different (at least $P < 0.05$; a > b > c; one way ANOVA followed by the Bonferroni or T2 Tamhane *post hoc* test). ED: rats fed the CFE-diet since the beginning of the study; D: rats fed the Chol-diet; DE: rats fed the CFE-diet when the diabetic state was confirmed; CFU, colony-forming units.

bacterial groups in feces of the three experimental groups. Diet significantly affected GM composition (ANOVA at least, $P = 0.015$).

ED rats presented significantly more concentration of *Bacteroides* spp. (9.1%), *Bifidobacterium spp.* (12.6%), *F. prausnitzii* (5%), and *Lactobacillus spp.* (4.7%). However, showed less *Blautia coccoides-Eubacterium rectale group* (−4.5%) and *Clostridium leptum* group (−4.6%) than the D group (all, $P < 0.05$). The DE group had less concentration of the *Blautia coccoides-Eubacterium rectale* group (−5.3%,) *Enterobacteriaceae* (−16.8%) and *Enterococcus spp.* (−15.7%) compared to its D counterparts (all, $P < 0.05$). *Bifidobacterium spp.* (15.3%) and *Enterobacteriaceae* (13%) contributed more to GM composition in the ED *vs.* DE rats (at least, $P = 0.008$).

3.3. Effect of CFE-RM on feces SCFAs levels

Fecal SCFAs results are shown in Table 5. Except for isovaleric acid, all SCFAs were significantly modified by diet (ANOVA at least $P = 0.009$). ED rats displayed higher content of total SCFAs, acetic (32.3%), propionic (31.1%), and butyric acids (52.2%) than D animals ($P < 0.05$). DE rats revealed significantly lower isobutyric (−25%) and valeric (−53.7%) acid levels than the D group ($P < 0.05$). ED rats showed higher

Table 5
Feces SCFAs levels of the three experimental groups.

SCFA (μmol/g feces)	ED	D	DE	ANOVA P
Acetic acid	27.67 ± 3.89a	20.92 ± 3.46b	21.27 ± 5.65ab	0.009
Propionic acid	6.66 ± 1.27a	5.08 ± 0.45b	5.29 ± 0.77ab	0.005
Butyric acid	1.75 ± 0.11a	1.15 ± 0.35b	1.23 ± 0.31b	0.001
Isobutyric acid	0.42 ± 0.09a	0.44 ± 0.07a	0.33 ± 0.04b	0.006
Valeric acid	0.48 ± 0.06a	0.54 ± 0.06a	0.25 ± 0.04b	0.002
Isovaleric acid	0.17 ± 0.05	0.18 ± 0.04	0.14 ± 0.03	NS

Labeled means in a row bearing different letters were significantly different (at least $P < 0.05$; a > b > c; one way ANOVA followed by the Bonferroni or T2 Tamhane *post hoc* test). ED: rats fed the CFE-diet since the beginning of the study; D: rats fed the Chol-diet; DE: rats fed the CFE-diet when the diabetic state was confirmed. NS, non-significant ($P > 0.05$).

butyric, isobutyric, and valeric acid fecal levels (29.7%, 27.3% and 92%, respectively; ANOVA at least, $P = 0.024$) than the DE rats. Representative chromatograms are shown in Supplemental Fig. S3.

3.4. Effect of CFE-RM on the proximal and distal colon morphology

To assess the protective effect of CFE-RM on the intestinal barrier, the morphometric parameters such as crypt density, crypt depth, the number of goblet cells and the proliferative/apoptosis balance in the colon were studied (Fig. 1).

Non-significant modifications at the proximal colon were observed. A distal section from D group showed disturbed colonic architecture. There were few shortened lined crypts with scanty goblet cells and diffuse inflammatory cell infiltrations showing decreased mucosa thickness. However, sections from ED and DE groups showed an apparent normal structure of the lining layers of the colon. CFE-RM significantly modified all distal morphometric parameters except the colonic density (ANOVA, at least $P < 0.001$). ED animals presented higher crypt depth, resulting in an overall higher mucosal thickness of the distal colon (ED *vs.* D, 20.4%; $P < 0.001$). Regarding goblet cells, morphometric analysis of PAS-stained sections showed a significantly higher number of goblet cells in ED rats than the D group (54.3%, $P < 0.001$). Further, ED rats showed inflammatory cell infiltrations in the lamina propria (Fig. 2A).

Changes in the colonic tissue homeostasis were measured evaluating the Colonocyte proliferation and apoptosis (Fig. 1C and D). Diet significantly affected proliferative/apoptosis balance in the distal section (ANOVA at least, $P = 0.007$). In all animals, PCNA-labelled nuclei were typically located at the lower half of the crypts, zone of proliferating cells (Fig. 1C). ED rats showed a significantly higher PCNA labeling in the distal colon compared to the D group (21.8%, $P < 0.001$). TUNEL-positive cells showed that apoptotic bodies were typically located at the top of the colonic epithelium crypts (Fig. 2D). The distal colon of ED and DE rats showed a higher number of apoptotic cells than the D counterparts (163.4% and 76.6%; $P < 0.001$ and $P = 0.011$, respectively).

3.5. Effect of CFE-RM on tight junctions of rat proximal and distal colonic mucosa

The protective effects of CFE-RM on paracellular permeability were determined to measure the TJs zonula occludens-1 (ZO-1) and occludin immunoreactivity levels in both proximal and distal colonic sections (Fig. 2).

A CFE-Chol-diet *vs.* Chol-diet induced a differential effect on ZO-1 and occludin proteins expression (ANOVA, at least $P < 0.001$). The ZO-1 immunostaining (Fig. 2A) was diffuse and less intensive in distal colon of ED and DE in comparison with D rats (−51.1% and −30.0%,

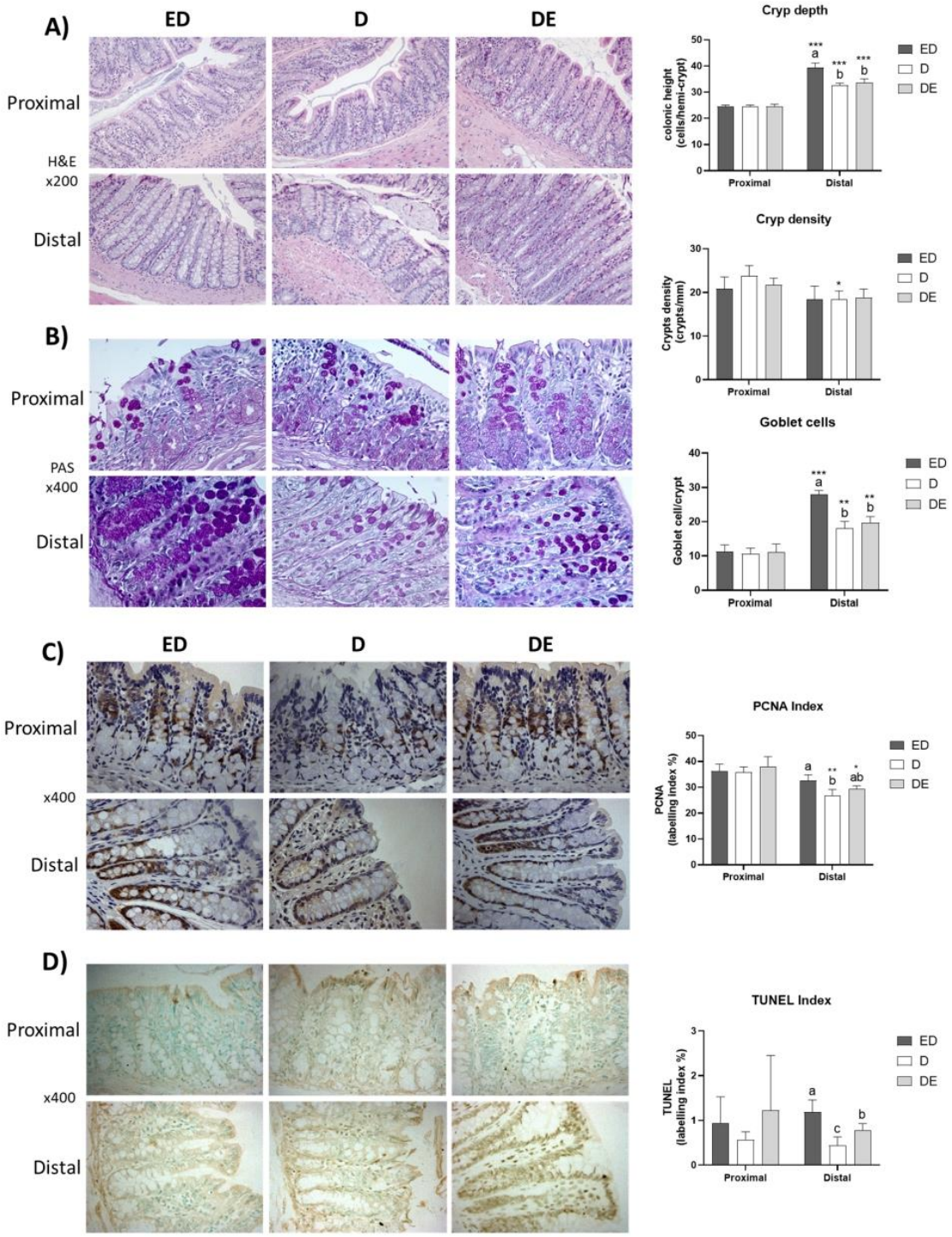

Fig. 1. Effect of CFE-RM on proximal and distal colon morphology. Data are mean ± SD (n = 8/group). ED: rats fed the CFE-diet since the beginning of the study; D: rats fed the Chol-diet; DE: rats fed the CFE-diet when the diabetic state was confirmed. (A) Histochemical H&E–stained cross section of proximal and distal colon (×200), Crypt depth (number of cells per hemicrypts) and crypt density (number of crypts/mm). (B) Histochemical PAS staining images (×400) and number of goblet cells per crypt. (C) Percentage of PCNA-positive cells (×400). (D) Colonic epithelial apoptosis as revealed by TUNEL assay (×400). Labeled means for a variable with different letters were significantly different ($P < 0.05$; a > b > c; ANOVA followed by the Bonferroni or T2 Tamhane *post hoc* test). Differences between proximal and distal colon sections of the same study group were indicated with asterisk (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; unpaired Student's *t*-test). H&E, hematoxylin and eosin; PAS, periodic Acid-Schiff; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

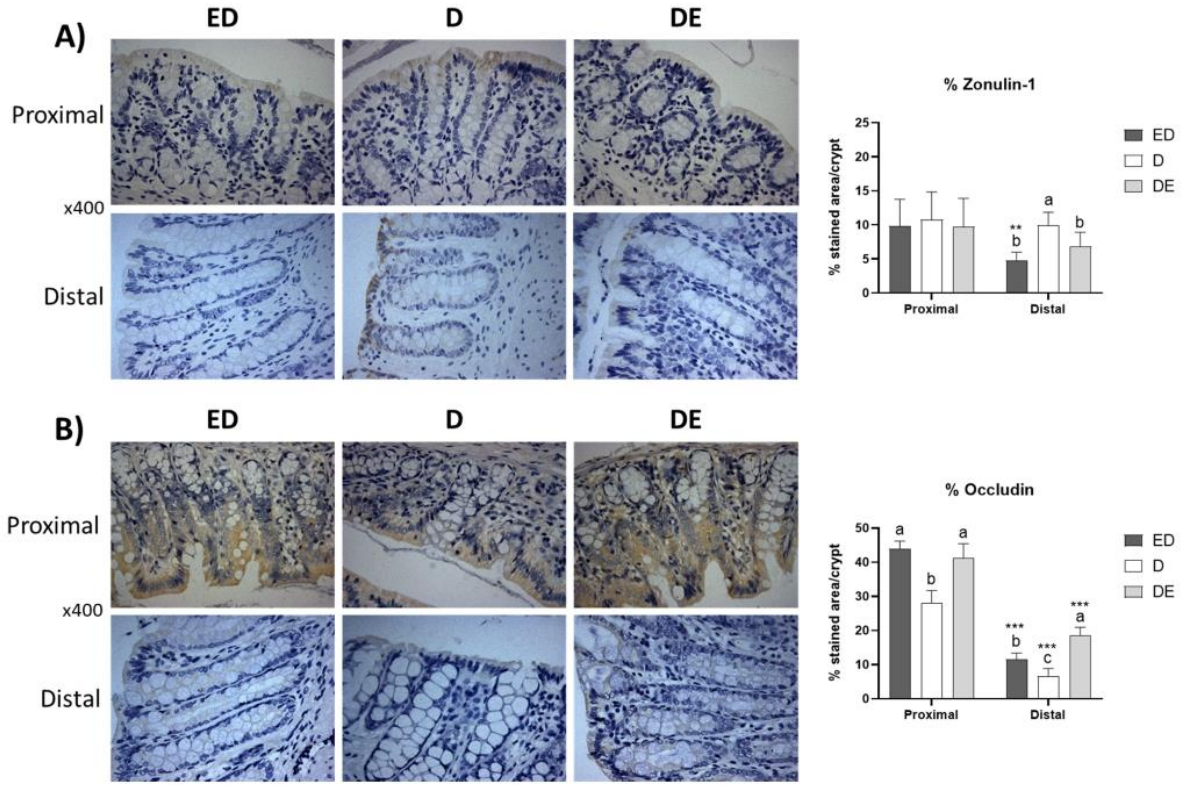

Fig. 2. Effect of CFE-RM on TJs in proximal and distal colon. Data are mean ± SD (n = 8/group). ED: rats fed the CFE-diet since the beginning of the study; D: rats fed the Chol-diet; DE: rats fed the CFE-diet when the diabetic state was confirmed. (A) ZO-1 immunohistochemical staining quantification per crypt (×400). (B) Occludin immunohistochemical staining quantification per crypt (×400). Labeled means for a variable with different letters were significantly different ($P < 0.05$; a > b > c; ANOVA followed by the Bonferroni or T2 Tamhane *post hoc* test). Differences between proximal and distal colon sections of the same study group were indicated with asterisk (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; unpaired Student's *t*-test). TJ, tight junctions; ZO-1, zonula occludens-1.

respectively; at least P = 0.013). This staining was mainly located at the top of the crypts (Fig. 2A). However, ED and DE rats showed an occludin strong immunostaining in the cytoplasm of the epithelial cells along the crypts in the proximal colon and at the top of the crypts in the distal colon (Fig. 2B). ED and DE rats presented higher occludin staining than D rats at the proximal section (57.5% and 47.3%, respectively; all $P < 0.001$) and at the distal portion (72.6% and 177.1%; all $P < 0.001$) (Fig. 2B).

3.6. Correlation of metabolic parameters, gut microbiota, SCFAs and distal colon integrity

Pearson product-moment correlations were performed to establish the relationships between metabolic parameters, GM, SCFAs and distal colon integrity. Fig. 3 summarizes, highlighting with asterisks, all significant correlations found.

Strong positive associations were found between *Lactobacillus* spp. and acetate and butyrate levels ($P < 0.01$), *F. prausnitzii* and butyrate ($P < 0.05$), *Bifidobacterium spp.* and propionate and butyrate ($P < 0.05$). The main SCFAs (acetate, propionate, and butyrate) correlated positive and significantly with crypt depth, positive goblet cells, PCNA index and TUNEL index ($P < 0.01$). ZO-1 strongly and negatively correlated with insulin, *Bacteroides* spp., *Lactobacillus* spp., *F. prausnitzii,* crypt depth, positive goblet cells, PCNA index, and TUNEL index.

3.7. Effect of CFE-RM on colonic markers concurrence

Table 6 shows the tertile distribution for the three experimental

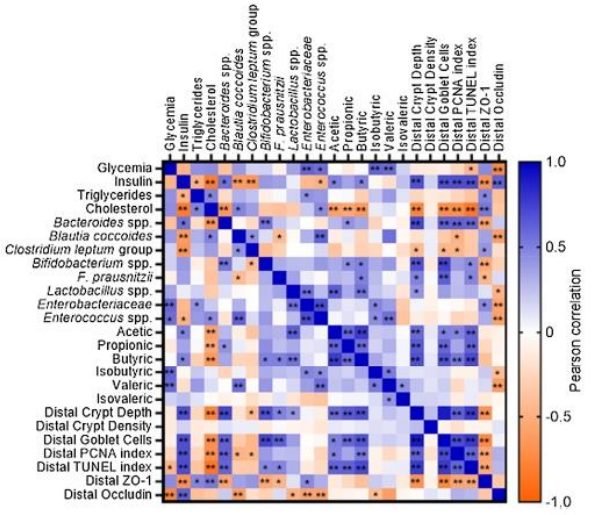

Fig. 3. Correlation analysis between metabolic parameters, gut microbiota, SCFAs levels and distal colon integrity in late-stage T2DM rats. Pearson product-moment correlation values were used for the matrix. The color intensity of the heatmap represents the association degree. *$P \leq 0.05$; **$P \leq 0.01$.

Table 6
Colonic markers concurrence in the three experimental groups.

Item and definition	Score	Rats with results, % ED	D	DE	Significance
GM_{Index}					
First tertile	1	0	4	1	$\chi^2 = 13.47$
Second tertile	2	2	4	5	P = 0.009
Third tertile	3	6	0	2	
Average score		2.75	1.5	2.13	
Median (ITR)	Range 4–12	10.5 (1) a	7 (2)b	7 (1)b	P' = 0.008
$SCFAs_{index}$					
First tertile	1	0	2	2	$\chi^2 = 11.0$
Second tertile	2	2	6	4	P = 0.027
Third tertile	3	6	0	2	
Average score		2.75	1.75	2	
Median (ITR)	Range 6–18	15 (1)a	10.5 (1) b	10 (2)b	P' = 0.004
CM_{Index}					
First tertile	1	0	7	1	$\chi^2 = 24.27$
Second tertile	2	2	1	6	P < 0.0001
Third tertile	3	6	0	1	
Average score		2.75	1.12	2	
Median (ITR)	Range 5–15	14 (1)a	8 (2)b	9.5 (3)b	P' = 0.004
TJ_{index}					
First tertile	1	0	6	0	$\chi^2 = 17.92$
Second tertile	2	3	2	2	P = 0.001
Third tertile	3	5	0	6	
Average score		2.63	1.25	2.75	
Median (ITR)	Range 2–6	5 (1)a	2 (0)b	5 (0)a	P' < 0.001
GC_{Index}					
First tertile	1	0	5	1	$\chi^2 = 30.40$
Second tertile	2	0	3	7	P < 0.0001
Third tertile	3	8	0	0	
Average score		3	1.38	1.88	
Median (ITR)	Range 17–51	43 (0)a	27 (5)c	32.5 (3) b	P' < 0.001

All data are rat distribution per group into tertiles except for median values (n = 8 rats/group). The average score was calculated considering the value 1, 2 and 3 for the tertiles 1st, 2nd and 3rd, respectively, considering the rat number into each tertile. The analysis was made by Kruskal-Wallis test followed by a non-parametric multiple comparison *post hoc* test (P' < 0.05; a > b > c) or chi-squared test as appropriate (P *value*). Labeled means in a row bearing different letters were significantly different. GM_{index} (*Bifidobacterium* spp., *F. prausnitzii*, *Lactobacillus* spp. and *Enterobacteriaceae*) was obtained considering the association of 4 species and their value calculated by adding the tertile value of each parameter (GM_{index} range, 4 to 12); total $SCFA_{index}$ (ramified plus non-ramified fatty acids ($SCFA_{index}$ range, 6 to 18); the colon morphology index CM_{index} (crypt depth, crypt density, positive goblet cells/crypt, PCNA index, TUNEL index) (CM_{index} range, 5 to 15); the TJ_{index} was calculated adding tertile values of occludin plus ZO-1 (TJ_{index} score range, 2 to 6); and the global colonic index (GC_{index}, considering a conjoint marker of 17 variables obtained from the addition of tertile values of GM_{index} + $SCFA_{index}$ + CM_{index} + TJ_{index} (GCi_{index} range from 17 to 51). For more information, see material and methods section. ED, rats fed the CFE-diet since the beginning of the study; D, rats fed the Chol-diet; DE, rats fed the CFE-diet when the diabetic state was confirmed. χ^2, chi-squared; CM_{index}, colon morphology index; GC_{index}, global colonic index; GM_{index}, gut microbiota index; ITR, intertertile range; SCFAs, short-chain fatty acids; $SCFAs_{index}$, short-chain fatty acids index; TJs, tight junctions; TJ_{index}, tight junctions index.

groups and the values for the different indexes obtained after combining single markers. All index values were significantly affected by diet (Kruskal Wallis test, at least P < 0.008). ED rats had the highest scores for GM_{index}, $SCFAs_{index}$; CM_{index} and the GC_{index} whereas the D group showed the lowest (significant differences, at least P = 0.025). Except for the TJ_{index}, all indexes differed in groups ED *vs*. DE (at least P < 0.05). Chi-squared tests suggest that the GC_{index} obtained combining 17 parameters, discriminated differences between the three groups. Thus, eight of eight rats in the ED group fit at the highest tertile, whereas five from eight rats in the D group fit within the first tertile and three into the second tertile. Seven rats from eight of the DE group fit into the second tertile whereas only one fit at the first tertile (P < 0.0001).

4. Discussion

Results described here show for the first time the preventive and curative positive effects of CFE-RM consumption on the GM-SCFAs-colonic barrier integrity interrelation in a late-stage T2DM model. As reported, late-stage T2DM was induced in Wistar rats by the conjoint action of Chol-diet and a single dose of STZ-NAD (Macho-González et al., 2019; Macho-González,López-Oliva, et al., 2020). The general characteristics exhibited by the T2DM control rats (D group) were like those found in humans and showed features of late-stage T2DM, such as impaired glucose tolerance with hyperglycemia, insulin deficiency by a mild pancreatic β-cell dysfunction without completely compromising insulin secretion, and dyslipemia (Macho-González et al., 2019). Results suggest that D group rats exhibited disturbed distal colon mucosa morphology, the downregulation of the goblet cell mucin producers and defects in epithelial TJs leading to mucosal-barrier integrity dysfunction. This would facilitate the translocation of luminal toxicity into the host (Cani et al., 2008).

A consensus exists that high-saturated fat diets strongly affect GM richness and variety, decreasing the *Bacteroidetes* amount and increasing *Firmicutes* and *Proteobacteria* in animals and humans (Cani et al., 2008; Hildebrandt et al., 2009; Mokkala, Houttu, Cansev, & Laitinen, 2019; Murphy et al., 2015). However, dietary fiber and polyphenol consumption has been postulated as a powerful therapeutic agent by modulating GM homeostasis and improving colonic health (Duda-Chodak, Tarko, Satora, & Sroka, 2015; Makki, Deehan, Walter, & Backhed, 2018; Zhao et al., 2018). Therefore, we first evaluated the preventive effect of CFE-RM consumption (ED group) *vs*. the D group on GM. *Bacteroides* spp., *Bifidobacterium* spp., *F. prausnitzii* and *Lactobacillus*, considered as beneficial bacteria, were more abundant in the ED than D rats. These results agree with others who tested high fiber and flavanol diets (Duda-Chodak et al., 2015; Wu et al., 2011), and PACs from red wine or blueberries (Anhe et al., 2015; Gil-Cardoso et al., 2016; Lee et al., 2018). Likewise, the highest colony-forming units (CFU) as CFU/g feces of *F. prausnitzii* was found in ED animals suggesting the importance of CFE in the T2DM prevention. In this study, results are relevant as *F. prausnitzii* has broadly demonstrated to have antiinflammatory properties and because its presence is frequently reduced among T2DM patients (Gurung et al., 2020; Qin et al., 2012). In addition, lower amounts of bacteria from the *Clostridium leptum* group was observed in ED animals, agreeing with previous reported results (Duda-Chodak et al., 2015). Based on general and well-accepted relationships between GM and colonic health effects (Kolodziejczyk, Zheng, & Elinav, 2019; Lau & Vaziri, 2019; Qin et al., 2012; Saldaña et al., 2020), beneficial bacteria *Bifidobacterium* spp., *F. prausnitzii*, and *Lactobacillus* spp., and non-beneficial *Enterobacteriaceae* were conjointly studied integrating the GM_{index}. ED rats showed a 50% GM_{index} higher than their D counterparts, suggesting the usefulness of this bacterial index. Thus, perhaps the GM changes observed were related to the following (a) the CFE potent inhibitory effect on carbohydrate digestion and absorption previously observed (Macho-González et al., 2019; Macho-González et al., 2018), allowing carbohydrates to reach the colon more abundantly and serving as substrate for specific bacteria; (b) CFE, besides playing an important prebiotic effect on GM under physiological conditions, increases growth and bacterial diversity (Anhe et al., 2015; Cires, Wong, Carrasco-Pozo, & Gotteland, 2016; Macho-González, Garcimartín, et al., 2020); and c) CFE serves as a substrate during its metabolism, producing SCFAs and available phenolic compounds that can exert beneficial local and systemic effects (Gurung et al., 2020; Macho-González, Garcimartín, et al., 2020; Ou et al., 2014). In relation with premises a, b, and c, fecal acetic, propionic and butyric levels were higher in group ED than in D, supporting previous results where fiber and polyphenol consumption

increased SCFAs production (Cires et al., 2016; Kolodziejczyk et al., 2019). In this study, levels could be, at least partially, due to the higher presence of *Lactobacillus* spp., *Bifidobacterium spp*. as acetate and propionate producers, and to *F. prausnitzii* as a butyrate producer (Feng, Ao, & Peng, 2018). In fact, positive and significant correlations between SCFAs and those bacterial abundances were observed (Fig. 3), showing relevant indirect associations. Thus, higher SCFAs levels could be involved in the improvement of glucose homeostasis recently reported in the same T2DM model (Macho-González,López-Oliva, et al., 2020), because some have observed that acetic and propionic acids can stimulate the pancreatic β-cell proliferation and induce insulin, GIP, and GLP1 production (Mandaliya & Seshadri, 2019). In contrast, butyrate is considered the most important SCFAs for colonocyte metabolism, which has been amply associated with colonic barrier integrity improvement (Liu et al., 2018; McNabney & Henagan, 2017). CFE-RM inclusion, as a T2DM preventive treatment, improved distal colon architecture in ED group, displaying greater mucosal thickness and crypt depth compared with group D. This study also shows that feeding CFE-RM induced a higher number (54.3%) of goblet cells in the distal colon of ED than in D animals, suggesting a stimulatory effect on goblet cell function while also showing higher cell differentiation and strengthening of the gut barrier. These results are in line with the effect on the secretory activity of intestinal goblet cells described in polyphenols and fiber fed animals (Barcelo et al., 2000). However, the potential role of *Lactobacillus* spp. and SCFAs on the goblet cells proliferation (Sicard, Le Bihan, Vogeleer, Jacques, & Harel, 2017) should not be discarded, as a positive and significant relationship was found between butyrate and acetate fecal levels and goblet cell abundance (Fig. 3). The intestinal epithelial barrier integrity also depends on a delicate balance between cellular proliferation and apoptosis. Colonocytes are constantly being removed and replaced guided by an active proliferating layer at the base of the Lieberkühn crypts (Rohr, Narasimhulu, Rudeski-Rohr, & Parthasarathy, 2020). Balance loss between proliferation and apoptosis, occurring after consuming high-fat diets or in some chronic pathologies, induces "holes" formation in the mucosa which increases intestinal paracellular permeability (Rohr et al., 2020). The microscopic examination of the colonic tissue sections showed a greater cell proliferation given by higher PCNA positive cell levels in the distal colonic mucosa of the group ED *vs.* D rats. In parallel, apoptotic cells, as determined by TUNEL-positive cells, were significantly higher in this same group, suggesting increased cell turnover. The positive relationships between butyrate, crypt depth, goblet cells, PCNA, and TUNEL indices found suggest a positive role of butyric acid on colonic homeostasis. Thus, butyrate deserves special attention as it is the primary source of energy for colonocytes (Liu et al., 2018). This fatty acid has been shown to promote epithelial turnover and maintain the colonic intestinal barrier, and to reduce the number and size of the aberrant crypt focus (Canani et al., 2011). Therefore, the higher fecal butyrate concentration of ED rats would partly suggest a more adequate rate of cell renewal, indicating better colonic health on these rats.

ZO-1, occludins and claudins, are crucial for the maintenance of epithelial barrier integrity (de Kort, Keszthelyi, & Masclee, 2011). ED rats displayed 51.1% lower ZO-1 levels in the distal colon than the D rats, suggesting an increase of the barrier integrity. These data are in line with those described by Palacios et al. (2020), who found a decrease in plasma ZO-1 levels in well-controlled T2DM patients. Furthermore, they could indicate of a greater ZO-1 release to serum, a fact associated with increased intestinal permeability in overweight and T2DM patients (Sánchez-Alcoholado et al., 2017). Interestingly, ZO-1 levels were positively associated with fasting glycemia, cholesterolemia and triglyceridemia, but negatively with insulin levels, crypt depth, goblet cells, PCNA, and TUNEL indices (Fig. 3). High-fat diet chronic consumption negatively regulates the TJs expression and inhibits the mucin secretion by goblet cells, allowing the entrance of luminal components to the lamina propria (Rohr et al., 2020). However, studies on ZO-1 in diabetic models are contradictory, because some authors describe a decrease in ZO-1 as an inducer of intestinal hyperpermeability (Cani et al., 2008), whereas others report that ZO-1 overexpression of this protein promotes the loss of the intestinal barrier, proposing serum ZO-1 levels as a biomarker for T2DM (Sturgeon & Fasano, 2016). Therefore, more studies are necessary to support the involvement of ZO-1 in T2DM. However, occludin is a binding protein that provides structural integrity to TJs. High occludin levels have been proposed as colonic barrier health markers (Chelakkot, Ghim, & Ryu, 2018). CFE-RM consumption as a preventive treatment (ED group) appears to strengthen TJs by increasing occludin levels in both colonic segments. Our results agree with those observed in the proximal colon of healthy rats fed grape-seed PACs (Goodrich et al., 2012). Furthermore, the results are also reinforced by the TJ_{index}, whose average score was 2.63 (62.5% of ED rats were in the highest score) whereas the average score of group D rats was 1.25 (75% of D rats were in the lowest tertile).

On the other hand, DE rats did not present the same modifications as ED *vs.* the D control rats for GM richness and SCFAs production or GM_{index} and $SCFA_{index}$. These results are relevant because it shows that the prebiotic effect attributed in a generalized way to fiber and PACs depends on the pathophysiological situation and should be contemplated. However, it reduced the presence of species considered potentially harmful, such as *Enterobacteriaceae* and *Enterococcus* spp. (Lambeth et al., 2015). Besides, DE rats also showed lower abundance of *Blautia coccoides-Eubacterium rectale* group. A positive association between the T2DM degree development, glycemic control and *Blautia coccoides-Eubacterium rectale* group levels has been suggested (Egshatyan et al., 2016), which follows our results, as CFE-Chol-diet fed rats showed better glycemic control, with lower glucose and higher insulin levels than group D rats (Macho-González,López-Oliva, et al., 2020). This is also supported by the significant correlations found between those bacteria and glucose homeostasis markers (Fig. 3); GM composition determines the SCFAs production (Feng et al., 2018; Mandaliya & Seshadri, 2019). Although acetic, propionic and butyric acids did not change, a significant decrease in isobutyric levels were observed in the DE group *vs.* D one, SCFAs levels have been associated with lipemia, with hypercholesterolemic patients presenting higher isobutiric levels (Granado-Serrano et al., 2019). In line with those results, lipemia was lower in group DE than in D rats (Macho-González et al., 2019). Furthermore, DE rats showed much higher occludin expression than the group D rats, both in the proximal and distal colon (47.3% and 177.1%; respectively). As previously mentioned, the average score of the TJ_{index} was 2.63 in ED and 2.75 in DE rats, suggesting a higher colonic integrity in CFE-RM fed rats, because the higher occludin levels are, the stronger the colonic barrier is. These results highlight the capacity of CFE-RM regulating TJs, which are crucial in barrier formation and maintenance (Rohr et al., 2020). Therefore, it can be hypothesized that CFE-RM curative treatment induces local action on the colonic mucosa to protect against the deleterious effects of Chol-diet-STZ-NAD, although more studies are necessary to confirm this. Despite not observing large changes in GM and SCFAs, the GC_{index} joining markers reveal a significant improvement on the colonic pathological status of group DE *vs.* D.

Because the GM-SCFAs-colonic barrier integrity interrelations in T2DM models are scarce, results in this study are novel and relevant, helping to understand the complex mechanisms in T2DM and the importance of including functional ingredients in meat, as a nutritional strategy for this disease. Nonetheless, this study has limitations: (1) only one dose of CFE was tested; (2) few bacterial species/genera have been analyzed in the GM study; (3) the study lasted only 8 weeks; (4) the study was conducted only in males; and (5) non-positive antidiabetic drug groups (Metformin or Glibenclamide) were included.

In summary, CFE-RM consumption as a preventive treatment (since the beginning of the study and before STZ-NAD diabetes induction) partially blocked the conjoint Chol-diet-STZ-NAD deleterious effects on GM richness, SCFAs production and colonic barrier integrity. On the other hand, CFE-RM as a curative treatment (after STZ-NAD diabetes induction) showed discreet changes of GM and SCFAs modulating

mucosal turnover and TJs levels on the distal colonic mucosa. The combination of selected bacterial genus/species (GM_{index}); ramified plus non-ramified fatty acids ($SCFA_{index}$); colonic morphology markers (CM_{index}); and TJ proteins (TJ_{ndex}) gave a global colonic score (GC_{index}) that differentiated the colonic health status of the experimental groups. In a scale with a minimum quotation of 17 and a maximum of 51, ED rats display a score of 43, DE rats, which consumed CFE-Chol-diet after the T2DM induction, showed a score of 32.5; whereas the control T2DM rats scored 27.

These promising results support that (a) CFE-RM development is a good strategy to increase fiber and PACs intake in the population; (b) CFE-RM consumption can contribute to slow down the T2DM progress, by holding adequate colonic health and better global results with the prevention strategy (ED) than with the curative (DE). However, future studies are needed to confirm the fiber and PACs beneficial effects on GM under different pathophysiological conditions.

CRediT authorship contribution statement

Adrián Macho-González: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Alba Garcimartín:** Conceptualization, Methodology, Investigation, Supervision, Writing - original draft, Writing - review & editing. **Noemí Redondo:** Investigation, Formal analysis. **Susana Cofrades:** Formal analysis, Methodology, Funding acquisition. **Sara Bastida:** Resources, Writing - review & editing. **Esther Nova:** Formal analysis, Writing - review & editing. **Juana Benedí:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Francisco J. Sánchez-Muniz:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition. **Ascensión Marcos:** Supervision, Writing - review & editing. **M. Elvira López-Oliva:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110124.

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Publicación 11

Carob Fruit Extract-enriched meat improves colonic antioxidant status in late stage T2DM rats

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Antecedentes: A pesar de que la DMT2 presenta un origen multifactorial, cada vez son más los estudios que señalan al estrés oxidativo como un agente esencial en su desarrollo (Wright y cols., 2006). Las alteraciones propias de la patología como la hiperglucemia postprandial pueden contribuir al desequilibrio del estatus antioxidante al producir una elevada cantidad de ROS (Wright y cols., 2006). A su vez, la dieta es una fuente continua de compuestos de oxidación, capaces de alterar la integridad intestinal e inducir estrés oxidativo (Li y cols., 2019), aunque la evidencia es muy escasa a nivel colónico. Concretamente, la carne y los productos cárnicos se han asociado con un aumento del estrés oxidativo y el desarrollo de patologías crónicas (Artículo 4). Sin embargo, la formulación de cárnicos funcionales con compuestos bioactivos con potencial antioxidante, como el caso del CFE, pueden ser una alternativa adecuada para paliar estos efectos adversos (Artículo 5).

Hipótesis: El consumo de un cárnico enriquecido en CFE mejora el estatus antioxidante colónico en un modelo de estado avanzado de DMT2.

Resultados: La caracterización del tipo de PACs contenidas en el CFE mediante resonancia magnética nuclear (RMN) reveló una elevada presencia de prodelfinidina B3 y prodelfinidina C2. Asimismo, los espectros de ^{1}H RMN mostraron un predominio de estructuras poliméricas de 3 a 9 unidades. El consumo del cárnico enriquecido en CFE, tanto bajo el punto de vista preventivo como terapéutico, indujo una mayor expresión y actividad de la maquinaria antioxidante a nivel de la mucosa proximal en comparación con el grupo control D. La mayor expresión pNrf2^{ser40} observada en la mucosa proximal de los grupos ED y DE *vs*. D, parece ser la causa principal que modula la activación de la maquinaria antioxidante. En relación con la defensa antioxidante no enzimática, el grupo ED mostró mayores niveles de GSH y menores de GSSG en relación con el control D en la mucosa colónica proximal, presentando un menor índice redox. Por el contrario, el grupo DE redujo notablemente los niveles de GSSG de la mucosa proximal, estableciendo diferencias significativas con ED y D.

Conclusiones: El CFE presenta un elevado contenido de prodelfinidina B3 y prodelfinidina C2 en su composición, conformando mayoritariamente estructuras de 3 a 9 unidades. Su consumo incluido en un cárnico funcional, tanto como estrategia preventiva como curativa, incrementa la expresión y actividad de la maquinaria antioxidante, principalmente a nivel de la mucosa colónica proximal, al inducir la translocación de Nrf2 al núcleo. Por tanto, el consumo de cárnicos funcionales enriquecidos en CFE podría ser una estrategia nutricional eficaz para mejorar el estatus antioxidante intestinal de personas con DMT2.

Carob Fruit Extract-enriched meat improves colonic antioxidant status in late-stage T2DM rats

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Running title: Colonic antioxidant effect of carob fruit extract

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Hipócrates

V. DISCUSIÓN INTEGRADORA

El avance del conocimiento en la interacción dieta-salud ha hecho que la nutrición no solo se entienda como la mera acción de nutrir, sino que se interprete como una potente herramienta para prevenir y ralentizar el progreso de las enfermedades crónicas (Bennett y cols., 2015).

Esta Tesis Doctoral se ha desarrollado con el fin de conocer los efectos del consumo del extracto de algarroba (CFE) en diferentes modelos de rata y caracterizar los mecanismos moleculares responsables de los mismos. Para ello, se han realizado tres experimentos en ratas Wistar: el primero de ellos en ratas jóvenes y sanas para verificar los efectos del CFE sobre la digestión y absorción de hidratos de carbono y grasas; y posteriormente en dos modelos diferentes de DMT2 en los que se evalúan los efectos del consumo crónico de CFE como ingrediente funcional incluido en una matriz cárnica. Los tres experimentos realizados ponen de manifiesto que el CFE presenta un potente efecto antidiabético a través de diferentes mecanismos, entre los que se encuentran la modificación directa de la digestión, absorción y metabolismo de macronutrientes, así como efectos nutrigenómicos que afectan a marcadores importantes en la fisiopatología de la DMT2.

Esta sección se subdivide en subapartados que intentan responder a los objetivos planteados, donde se discuten en conjunto los resultados más importantes incluidos en esta Tesis Doctoral intentando integrar y relacionar el conjunto de los resultados y así poder emitir de forma neta unas conclusiones.

5.1. Efecto a corto plazo del consumo de extracto de algarroba sobre la digestión y absorción de hidratos de carbono y grasas

La capacidad de los polifenoles para modular la digestión y absorción de nutrientes, especialmente hidratos de carbono y grasa, ha sido ampliamente descrita (Bahadoran y cols., 2013; Hanhineva y cols., 2010). Por ello, en primer lugar, se quiso confirmar, tanto en estudios *in vitro* como *in vivo* (**Artículos 1 y 2**), que el CFE puede interferir sobre dichos procesos.

Inicialmente se valoró el efecto del CFE sobre la enzima α-glucosidasa (actividad maltasa), una de las enzimas clave en la digestión de los hidratos de carbono al hidrolizar la maltosa en dos glucosas y permitir su posterior absorción intestinal. El CFE produjo un efecto inhibitorio de forma dosis dependiente, describiéndose por primera vez en la bibliografía internacional que este extracto ejerce una inhibición enzimática de tipo competitivo (**Artículo 1**). La marcada inhibición obtenida (superior al 50%) para la dosis máxima ensayada parece deberse a la composición del CFE y al

tipo de PACs que contiene, ya que tal y como describe Xiao y cols. (2013), el grado de polimerización y el tipo de PACs condicionan el grado de inhibición de la enzima α-glucosidasa, sugiriendo un mayor efecto para estructuras poliméricas. La estructura y tipo de PACs descritas en el **artículo 11** (prodelfinidina B3 y prodelfinidina C2 conformando estructuras de 3 a 9 unidades) (Figura 15), y en consonancia con lo recogido en las patentes WO2004/014150 y WO2006/000551 (Ruiz-Roso y cols., 2006; Ruiz-Roso y cols., 2004), muestra un alto grado de polimerización que, junto con la elevada presencia de grupos hidroxilo de los flavan-3-ol, explicarían el marcado grado de inhibición de la digestión de los hidratos de carbono (Lee y cols., 2007). De forma paralela comprobamos que este mismo extracto produce *in vitro* una inhibición dosis-dependiente de la actividad de la lipasa pancreática, enzima que hidroliza los triglicéridos a 2-monoglicéridos y ácidos grasos libres (**Artículo 2**). Las PACs se han definido como potentes inhibidoras de la digestión de los lípidos, estableciendo una asociación significativa entre el grado de inhibición y grado de polimerización (Kimura y cols., 2011). Sin embargo, la importancia del grado de polimerización de las PACs sobre la actividad de la lipasa no resulta tan determinante como en lo expuesto para la enzima α-glucosidasa.

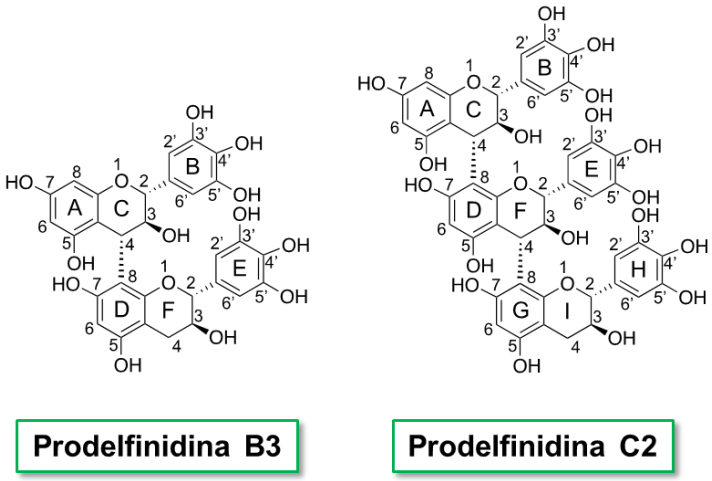

Figura 15. Estructuras de las PACs identificadas en el CFE.

CFE, extracto de algarroba; PACs, proantocianidinas.

Una vez comprobada la efectividad inhibitoria del CFE sobre algunas enzimas clave de la digestión de los hidratos de carbono y las grasas, se evaluó su efecto *in vivo* en dos estudios postprandiales (agudo y subcrónico) en ratas Wistar jóvenes a las que se administró 1,5mL de una mezcla de glucosa (250mg), aceite de oliva y CFE (**Artículos 1 y 2**). La rata ha sido considerada como un modelo adecuado para estudios de digestibilidad (Steingoetter y cols., 2019). Dada la implicación de la glucemia y lipemia postprandiales en el desarrollo de ECV y DMT2, y que muchas de

las terapias nutricionales y farmacológicas se dirigen a minimizar su impacto y duración (Ceriello, 2005; Ceriello y cols., 2002; Chan y cols., 2013), se evaluó el efecto potencial del CFE sobre ellas. Los resultados *in vivo* revelaron una disminución de la glucemia postprandial tras una única administración de CFE (estudio agudo), siendo más pronunciados sus efectos hipoglucemiantes tras una semana consumiendo dicho compuesto (estudio subcrónico) (Figura 16). Como se ha descrito, el CFE no solo está compuesto por PACs, sino que también presenta entre un 74−84% de fibra dietética total, de la que aproximadamente un 70% es fibra insoluble (Ruiz-Roso y cols., 2004). Por tanto, los efectos observados en el estudio postprandial agudo, puesto que se administra directamente glucosa, se deben asociar mayoritariamente al efecto arrastre o de retención de glucosa por la fibra, limitando su absorción (Ruiz-Roso y cols., 2010; Sánchez-Muniz, 2012). Este efecto fue confirmado en el experimento *in vitro* de difusión de glucosa, en el cual se observó una reducción de la difusión pasiva a través de una membrana porosa, que fue inversamente proporcional a la concentración de CFE estudiada (**Artículo 1**). Cabe remarcar en este punto que si se realizara una administración de hidratos de carbono complejos los efectos sobre la glucemia postprandial podrían ser incluso más evidentes, ya que posiblemente se encontraría un efecto sumatorio de la inhibición de la α-glucosidasa demostrada *in vitro* y atribuída a las PACs, con el efecto de la fibra insoluble recién comentado. Además, en el experimento subcrónico se encontró una marcada reducción de la glucemia postprandial en comparación con el estudio agudo, lo que sugiere la participación de mecanismos nutrigenómicos adicionales. Uno de estos mecanismos consistió en la reducción de los niveles del transportador de glucosa SGLT1 a nivel duodenal, que contribuye a la reducción de la absorción de glucosa (**Artículo 1**).

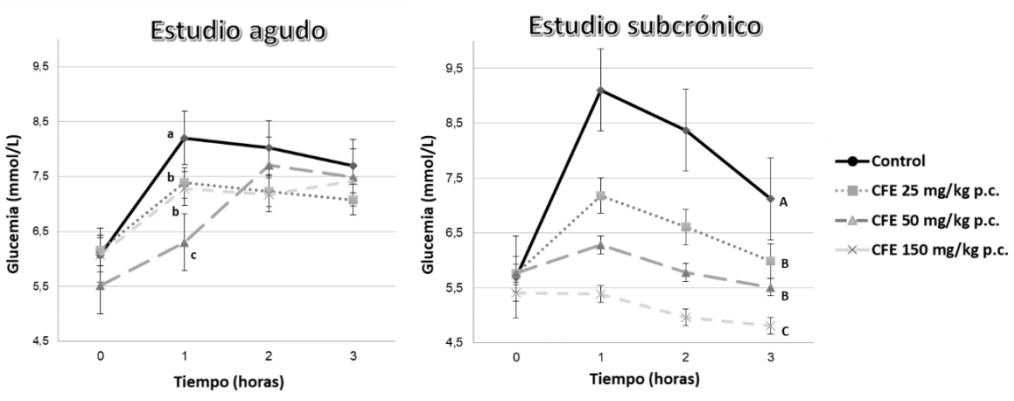

Figura 16. Efecto del consumo del CFE sobre la glucemia postprandial.

CFE, extracto de algarroba. Disponible en el artículo 1.

Por otra parte, la trigliceridemia postprandial se vio reducida ya desde la administración aguda de CFE, independientemente de la dosis ensayada, lo que

demuestra el potente efecto inhibidor del CFE sobre la actividad de la lipasa pancreática (**Artículo 2**) y el efecto arrastre o secuestrante de grasa. De nuevo, como se ha sugerido en el efecto hipoglucemiante, se establecería una sinergia entre las PACs (efecto sobre la lipasa) y la fibra insoluble (efecto arrastre) presentes en el CFE. El estudio subcrónico confirmó el efecto hipolipemiante. De hecho, al igual que en la glucemia, los resultados de la trigliceridemia y colesterolemia postprandiales fueron significativamente menores a los encontrados en el estudio agudo (Figura 17). Estos datos sugieren la implicación de mecanismos moduladores de la transcripción génica de enzimas y/o transportadores implicados en la digestión y absorción de grasas. Los efectos de las PACs tras administraciones repetidas, como ocurre en el experimento subcrónico, pueden ser desencadenados a nivel sistémico por los metabolitos activos de las PACs, liberados tras la fermentación colónica. Así, algunos autores como Bahadoran y cols. (2013) y Cires y cols. (2016) indican que son estos metabolitos activos de las PACs los responsables de los efectos nutrigenómicos. Por otra parte, las propiedades hipolipemiantes del CFE, demostradas previamente por otros autores, se han relacionado con su elevado contenido en fibra dietética y PACs (Goulas y cols., 2016; Ruiz-Roso y cols., 2010; Valero-Muñoz y cols., 2019), que promoverían: ralentización del vaciamiento gástrico e incremento del tránsito intestinal, lo que dificulta el acceso de las enzimas digestivas y reduce la digestión y absorción de lípidos (Rtibi y cols., 2017; Ruiz-Roso y cols., 2010). Para demostrar la implicación de estos mecanismos sobre la lipemia postprandial, se analizó la composición lipídica del lumen intestinal y de las heces. Los resultados coinciden con dicha hipótesis al observarse un mayor contenido de triglicéridos sin hidrolizar en el remanente intestinal y un incremento significativo de triglicéridos y colesterol en heces tras la administración de la dosis más elevada de CFE (**Artículo 2**).

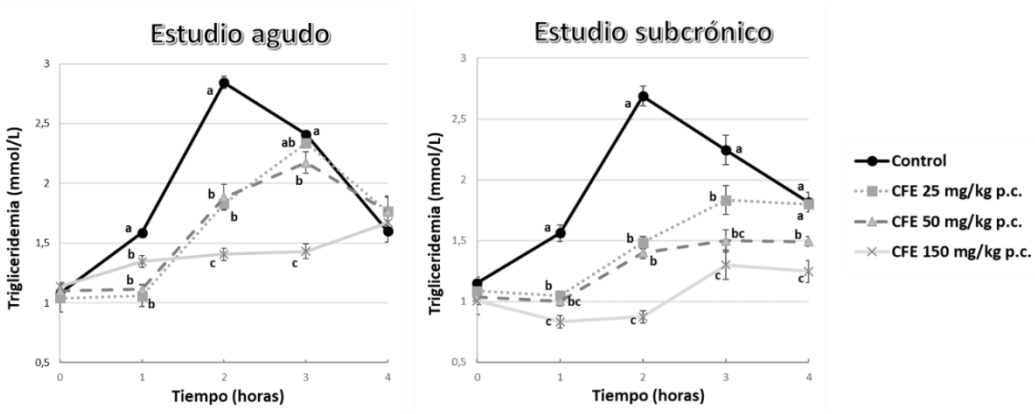

Figura 17. Efecto del consumo del CFE sobre la trigliceridemia postprandial.

CFE, extracto de algarroba; p.c., peso corporal. Disponible en el artículo 2.

Los efectos del consumo de CFE sobre el metabolismo postprandial le convierten en un candidato interesante para ser empleado como ingrediente en el diseño y formulación de alimentos funcionales (Figura 18). Para probar su viabilidad como ingrediente funcional, es necesario en un primer paso encontrar un sistema viable que permita vehiculizarlo sin que pierda sus propiedades; para finalizar posteriormente con la elección de una matriz adecuada y la evaluación científica de las propiedades saludables de dicho alimento. Solo siguiendo este algoritmo se podrá clasificar el producto dentro de la categoría de alimentos funcionales (**Artículos 3 y 5**) (Ashwell, 2002). Uno de los sistemas más empleados en la actualidad para el diseño de alimentos funcionales son las emulsiones, ya que establecen estructuras multicompartimentalizadas en las que se pueden incorporar los compuestos bioactivos con relativa facilidad (Jiménez-Colmenero, 2013). Por ello, se elaboraron emulsiones simples y gelificadas con diferentes proteínas emulsificantes (SC y WPI), añadiendo CFE como ingrediente funcional. Las características tecnológicas del sistema permitieron adicionar 3,9g de CFE por 100g de emulsión (**Artículo 3**), lo que aseguraba el cumplimiento del primer objetivo del diseño del alimento funcional, la viabilidad (Figura 18). Aunque los resultados reológicos obtenidos tienen gran importancia práctica para el diseño de los alimentos funcionales, debido al contenido y enfoque de la tesis, no se discutirán, aunque se pueden consultar ampliamente en el **artículo 3**. Con el objetivo de corroborar el efecto inhibitorio del CFE sobre la digestión de las grasas y el mantenimiento de sus propiedades antioxidantes tras ser incluido en la emulsión, se estudió el comportamiento de los sistemas formulados en un protocolo validado de digestión *in vitro*. Los resultados relativos a la digestión gastrointestinal *in vitro* revelaron que el efecto hipolipemiante del CFE está condicionado a la proteína emulsificante empleada en la formulación de estos sistemas, lo que justifica la relevancia de este tipo de estudios (**Artículo 3**). Así, en las emulsiones elaboradas con SC, el CFE no redujo la digestión lipídica en comparación con el control, lo que revelaría una pérdida de su capacidad inhibitoria de la lipasa pancreática. La causa de dicha pérdida puede ser la formación de complejos entre PACs y SC (Ma y cols., 2021; Prigent y cols., 2009), que no permitirían ejercer la acción esperada sobre dicha enzima. Por el contrario, las emulsiones con WPI y CFE sí mostraron los resultados esperados, corroborando el efecto inhiibitorio de la digestión de grasas observado en el experimento *in vivo* (**Artículo 2**). Por otra parte, en ambos sistemas (SC y WPI) se observó una importante liberación de PACs y el mantenimiento de sus propiedades antioxidantes. Si se extrapolan estos resultados a lo que ocurriría *in vivo*, la mayor liberación de CFE desde estos sistemas en el tubo digestivo protegería frente a la

posible formación de ROS durante la digestión y por tanto contribuiría a un mejor equilibrio antioxidante (**artículos 4, 5 y 11**).

Las emulsiones simples o gelificadas son unas de las formulaciones más utilizadas para mejorar el perfil graso de los alimentos cárnicos, a fin de aliviar algunos de los efectos perjudiciales asociados a su elevado consumo (**Artículo 4**) (Jiménez-Colmenero, 2007). Las matrices cárnicas brindan grandes oportunidades en el campo de los alimentos funcionales, ya que además de ser productos de elevada aceptación y consumo, son un vehículo excelente que permite incorporar compuestos bioactivos (Jiménez-Colmenero y cols., 2010), aportando propiedades beneficiosas que la carne *per se* no presenta (**Artículo 5**). Con el objetivo de identificar los beneficios de la elaboración de cárnicos funcionales, se realizó una revisión exhaustiva de los ingredientes con potencial antioxidante incorporados a productos cárnicos con mayor frecuencia; y posteriormente se evaluó su efectividad para limitar los procesos oxidativos típicos de los productos cárnicos que acontecen tanto en su procesado, como en su almacenamiento y finalmente en su cocinado. Asimismo, se evaluaron los estudios *in vivo* (animales y humanos) disponibles hasta la fecha, con el objetivo de conocer el efecto dual de su consumo, reduciendo la formación de ROS durante el proceso digestivo y mejorando el estatus antioxidante de los consumidores (**Artículo 5**). El análisis de la bibliografía reveló que el CFE no ha sido previamente incluido como ingrediente funcional en matrices cárnicas. Por tanto, y dado que el principal componente del CFE son las PACs, también quisimos ampliar los conocimientos realizando una revisión de los efectos derivados del consumo de este tipo de flavonoides, con especial énfasis en la microbiota, la permeabilidad intestinal y la inflamación (**Artículo 6**). Dado que parte de los efectos de las PACs se deben a los metabolitos activos derivados de la fermentación colónica, lo que ocurre en este órgano es especialmente trascendente (Rauf y cols., 2019). La revisión realizada permitió comprobar que, gracias a sus efectos prebióticos, antioxidantes y antiinflamatorios a nivel de colon, junto con otras propiedades importantes (anticancerígena, hipolipemiante y antidiabética), contribuyen a la prevención y/o mejora de numerosas patologías crónicas.

Una vez comprobada la viabilidad tecnológica para incluir el compuesto bioactivo de interés en un sistema lipídico y el mantenimiento de sus propiedades tras una digestión *in vitro* (**Artículo 3**), todo hace indicar que el diseño de un cárnico funcional enriquecido en CFE podría suponer una adecuada estrategia nutricional para controlar la digestión y absorción de hidratos de carbono y grasas. Además, también podría mejorar la estabilidad del propio cárnico prolongando su vida útil al retrasar los

procesos oxidativos que tienen lugar durante su procesado, almacenamiento y cocinado (**Artículos 4 y 5**). Por tanto, todos estos datos abren paso a la realización de estudios crónicos en los que se incluye el cárnico enriquecido en CFE, con el objetivo de demostrar las propiedades de mejora del estado de salud, reducción del riesgo de enfermedad, o ambas cosas derivadas de su consumo (Ashwell, 2002; Diplock y cols., 1999).

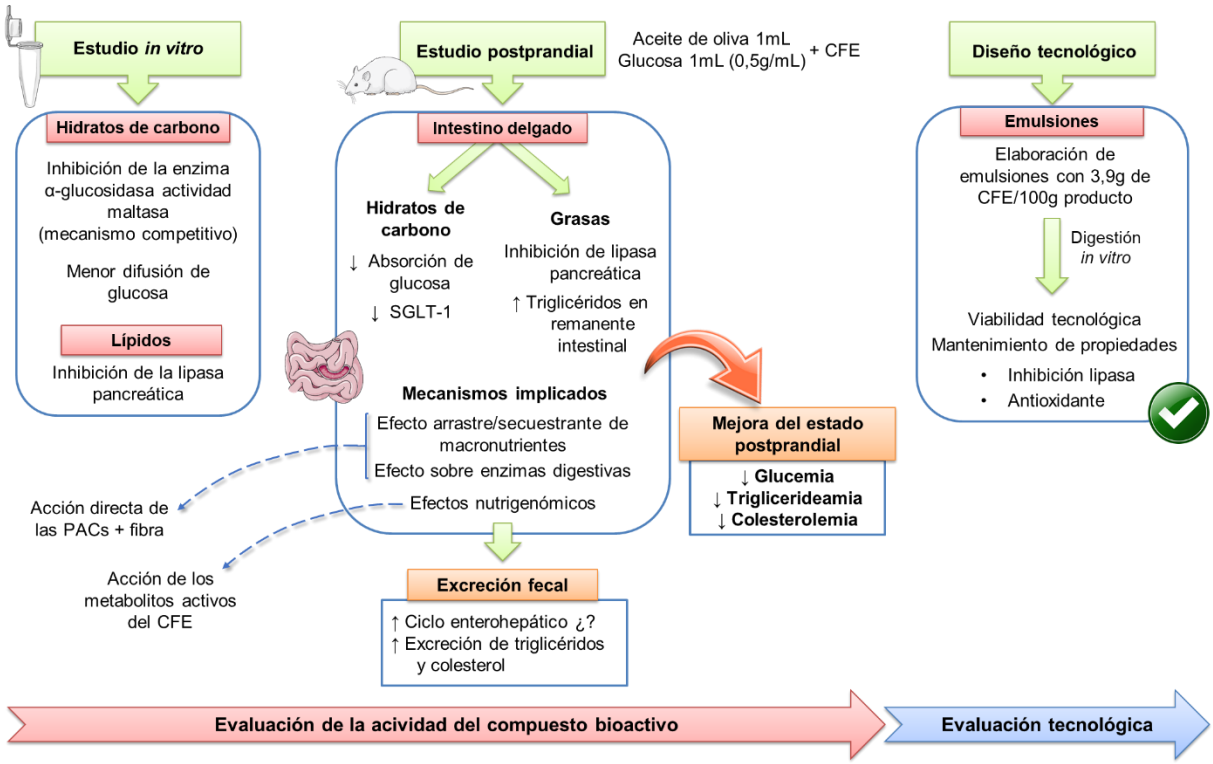

Figura 18. Esquema representativo del efecto del CFE sobre la digestión y absorción de hidratos de carbono y grasas.

CFE, extracto de algarroba; SGLT1, cotransportador de sodio-glucosa tipo 1.

5.2. Impacto del consumo un cárnico enriquecido en CFE sobre la fisiopatología de dos modelos murinos de DMT2

Los patrones dietéticos que se asocian a los pacientes con DMT2 se basan en un predominio de alimentos de origen animal, con elevado contenido en grasas saturadas, y una baja presencia de productos vegetales (Alhazmi y cols., 2014; McNaughton y cols., 2008). El consumo de dichos alimentos contribuye al desarrollo y progreso de la patología. Por ello, la piedra angular del tratamiento se basa en la modificación del estilo de vida con la mejora de los hábitos dietéticos (Neuenschwander y cols., 2019). Desgraciadamente, los datos demuestran la refractariedad de muchos pacientes con DMT2 a cambiar sus hábitos alimentarios, lo

que dificulta enormemente la implantación y seguimiento de una terapia nutricional correcta (Cradock y cols., 2017). En este contexto, los alimentos funcionales emergen como una posible alternativa a los cambios nutricionales drásticos, aportando compuestos bioactivos vehiculizados en matrices de elevado consumo. Por ello, la formulación de un cárnico funcional enriquecido en CFE resulta de especial interés para la población diabética, ya que constituye un alimento básico en sus dietas, no siendo necesaria una modificación de sus hábitos. Además, el CFE es fuente de fibra dietética (aproximadamente contiene un 70%) (Ruiz-Roso y cols., 2004), con lo que la adición de 3,9 g de CFE en el cárnico funcional podría contribuir a cumplir los objetivos nutricionales del consumo de fibra dietética (Saldaña y cols., 2020); y a su vez, presenta elevada cantidad de PACs que han demostrado propiedades antidiabéticas a diferentes niveles (**Artículo 6**) (Rauf y cols., 2019).

La selección de este ingrediente funcional se realizó en base a su eficacia a diferentes dosis (**Artículos 1 y 2**) y viabilidad tecnológica (**Artículo 3**), así como en diferentes formulaciones y aplicaciones (Goulas y cols., 2016; Rtibi y cols., 2017). No obstante, una vez diseñado y desarrollado el cárnico funcional conteniendo CFE, es necesario demostrar que el ingrediente funcional mantiene su funcionalidad de acuerdo a la finalidad para la que ha sido formulado (**Artículo 3 y 5**) (Jiménez-Colmenero y cols., 2010; Olmedilla-Alonso y cols., 2013), que en nuestro caso son sus propiedades antidiabéticas basadas en efectos hipoglucemiantes, hipolipemiantes, hepatoprotectores, prebióticos y antioxidantes. Para ello, en la presente Tesis Doctoral se han llevado a cabo dos experimentos que evalúan los efectos del consumo a largo plazo del cárnico funcional enriquecido en CFE en dos modelos de DMT2 correspondientes a las etapas inicial y avanzada de la patología.

5.2.1. Caracterización y justificación de idoneidad de los modelos animales empleados en los estudios crónicos

La DMT2 es una patología dinámica, cuyas características fenotípicas cambian a lo largo de su evolución. La alteración principal es la RI, que comprende tanto la ineficacia de dicha hormona para estimular la absorción de glucosa en el músculo esquelético y en el tejido adiposo, como la incapacidad para suprimir la producción de glucosa (gluconeogénesis) y destrucción de los depósitos de glucógeno (glucogenólisis); y fomentar la síntesis de glucógeno (glucogénesis) a nivel hepático (Fonseca, 2009; Franconi y cols., 2008). Estos factores conducen a la instauración de la DMT2, en la que se detecta hiperglucemia en ayunas como principal alteración analítica. En esta primera etapa de la patología el páncreas intenta contrarrestar la RI

liberando una mayor cantidad de insulina. Por ello, además de hiperglucemia, es habitual que curse con hiperinsulinemia desde estadios tempranos. Esta situación de gran demanda de insulina mantenida en el tiempo, sumada al posible progreso de la patología, llevan al páncreas a un agotamiento y muerte celular, caracterizado por la destrucción de las células β pancreáticas y la caída en la producción de insulina. Estos signos son característicos de una etapa avanzada de DMT2, en la que se detecta hiperglucemia e hipoinsulinemia (Fonseca, 2009) (Figura 19). Cuando esto ocurre los pacientes con DMT2 pasan a ser insulinodependientes, es decir, es necesario adicionar insulina exógena a su tratamiento antidiabético previo.

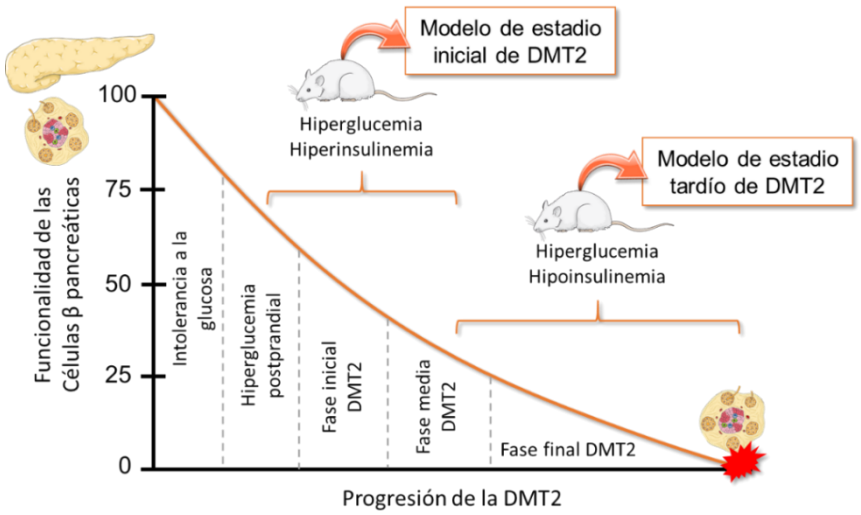

Figura 19. Progresión de la DMT2 en relación con la funcionalidad de las células β pancreáticas. DMT2, Diabetes Mellitus tipo 2

El diseño de diferentes modelos experimentales que repliquen los fenotipos de estos dos estadios de la patología, temprano y tardío, es crucial para evaluar con precisión la eficacia comparativa de los tratamientos antidiabéticos (farmacológicos y no farmacológicos). Tal y como sugieren algunos autores, el modelo murino (ratas y ratones, principalmente) parece ser el más adecuado para su estudio. Estos modelos brindan la oportunidad de evaluar el inicio, el desarrollo y la progresión de la enfermedad en función del diseño experimental, y contribuyen a la comprensión de los mecanismos moleculares subyacentes a la DMT2 (Franconi y cols., 2008; King, 2012) y permiten caracterizar los mecanismos de acción de compuestos bioactivos de aplicación sobre dicha patología. Por tanto, una vez introducidos los dos modelos de la DMT2 empleados en esta memoria de Tesis Doctoral, es necesario comentar y discutir la utilidad de su uso y las características fisiopatológicas que presentaron.

Es evidente la estrecha relación entre un elevado consumo de grasas, especialmente saturadas, y el desarrollo de RI y DMT2 (Rice Bradley, 2018). Es en

esta premisa en la que se basan la gran mayoría de protocolos para obtener modelos animales de DMT2 inducidos por dieta (Skovsø, 2014). De acuerdo con algunos autores, una dieta con un 30-50% de las kcal aportadas por la grasa, se considera una dieta alta en grasas (Gheibi y cols., 2017). Se ha descrito que este tipo de alimentación induce RI tras 3 semanas, aunque los modelos murinos obtenidos con este intervalo de tiempo no presentan un incremento en el porcentaje de grasa corporal. Por esta razón, recomiendan utilizar intervenciones nutricionales superiores a 5 semanas, con el objetivo de obtener un modelo con RI claramente definido y un incremento del tejido adiposo compatible con obesidad (Skovsø, 2014). Este tipo de modelos se caracterizan por hiperglucemia e hiperinsulinemia, simulando una etapa inicial de la DMT2 (Figura 19). No obstante, en ocasiones las condiciones del estudio requieren estadios más avanzados de la patología, en la cual se presenten alteraciones más notorias. En estos casos, la inducción exclusivamente con dieta demanda tiempos muy prolongados de intervención y no se plantea como la principal opción (Premilovac y cols., 2017; Sharma y cols., 2019; Skovsø, 2014). Para lograr un estadio más avanzado de DMT2, la forma más utilizada es la combinación de una dieta alta en grasa saturada y colesterol, con el objetivo de producir RI en los tejidos periféricos, junto con una dosis baja de STZ+NAD, que induce la destrucción selectiva de las células β pancreáticas sin agotar la función secretora de insulina (Reed y cols., 2000; Szkudelski, 2012). Esta combinación permite obtener un modelo murino con hiperglucemia e hipoinsulinemia, similar a los pacientes insulinodependientes con DMT2 de años de evolución que presentan disfunción pancreática (Fonseca, 2009). En nuestro caso, tal y como se ha comentado en los resultados del **capítulo 3**, seleccionamos una dieta alta en grasas con aproximadamente un 50% de las kcal totales de la dieta aportadas por este macronutriente, donde el 39% fueron AGS, a la cual se adicionó colesterol y ácido cólico (1,4% y 0,2%, respectivamente; Tabla 7). Numerosos estudios de nuestro grupo de investigación han demostrado que la incorporación de estos dos últimos componentes a la dieta de los animales, aceleran el desarrollo de NAFLD, alteración hepática más común en DMT2 y síndrome metabólico, y su progresión a NASH (Garcimartín y cols., 2017; González-Torres y cols., 2016). Además, favorecen el desarrollo de una dislipemia equiparable a la observada en pacientes con DMT2.

Teniendo en cuenta los objetivos perseguidos con el diseño de estos dos modelos animales, se describirán las alteraciones sistémicas más frecuentes que permiten categorizar el estado de la patología, para continuar posteriormente con una descripción breve de la fisiopatología que acontece a cada modelo animal (Tabla 10).

De acuerdo con (Skovsø, 2014), la primera característica de estos modelos es presentar hiperglucemia (glucosa en ayunas ≥126mg/dL), la cual se cumplió ampliamente en ambos casos, alcanzándose cifras de 250,8 mg/dL y 326 mg/dL para los animales C y D, respectivamente (**Artículos 7 y 9**). De forma paralela, los niveles de insulina son un factor clave a la hora de definir el estado de la DMT2 (Figura 19), ya que la hiperglucemia mantenida en el tiempo favorece la disfunción pancreática y compromete la secreción de insulina (Fonseca, 2009). A pesar de que no hay un punto de corte claramente establecido para definir la insulinemia en ratas, varios autores señalan que el intervalo de 9-12μIU/mL es el más común en los animales sanos (Koksal, 2015; Okoduwa y cols., 2017). En nuestro caso, el grupo C presentó una hiperinsulinemia marcada, con valores de 15,84μIU/mL, mientras que el D, como consecuencia de la inyección de STZ+NAD, reveló una clara hipoinsulinemia (5,41μIU/mL) (**Artículos 7 y 9**). De estos dos valores de glucemia e insulinemia se derivan los índices HOMA-IR, QUICKI y HOMA-β, los cuales permiten evaluar la homeostasis de la glucosa y son ampliamente utilizados para categorizar la DMT2 (Wallace y cols., 2004). A pesar de que el clamp hiperinsulinémico-euglucémico es considerado el estándar de oro para diagnosticar RI, el índice HOMA-IR y QUICKI son una buena alternativa para valorar sensibilidad a la insulina (Antunes y cols., 2016). Los valores de referencia para el diagnóstico de RI mediante HOMA-IR se sitúan por encima de 2 (Motamed y cols., 2016), encontrándose unas cifras de 9,79 para el grupo C, claramente indicativo de una situación de hiperglucemia e hiperinsulinemia; y de 4,57 para los animales D, resultado de la disfunción pancreática obtenida en este modelo de estadio avanzado de DMT2. De la misma forma, los valores del índice QUICKI se establecieron por debajo de 0,34, punto de corte fijado para definir RI, en ambos modelos animales, correspondiéndose con lo descrito anteriormente. Por último, el índice HOMA-β permite estimar en porcentaje la funcionalidad de las células β pancreáticas. Tal y como se esperaba, las ratas C mostraron un HOMA-β de 30,6, mientras que el grupo D presentó una cifra de 7,24 (**Artículos 7 y 9**).

En línea con lo discutido previamente, el grupo C no mostró alteraciones destacables en la histología del páncreas. Sin embargo, la evaluación histopatológica del páncreas del grupo D reveló una atrofia en los islotes de Langerhäns, con pequeñas vacuolas inflamadas en las células acinares (Figura 20), lo que explica los valores de HOMA-IR, QUICKI y HOMA-β (Cao y cols., 2016). Todas estas características permiten definir el estadio de la DMT2, encontrando dos fenotipos claramente diferenciados de acuerdo a otros autores (Gheibi y cols., 2017; Premilovac y cols., 2017; Skovsø, 2014).

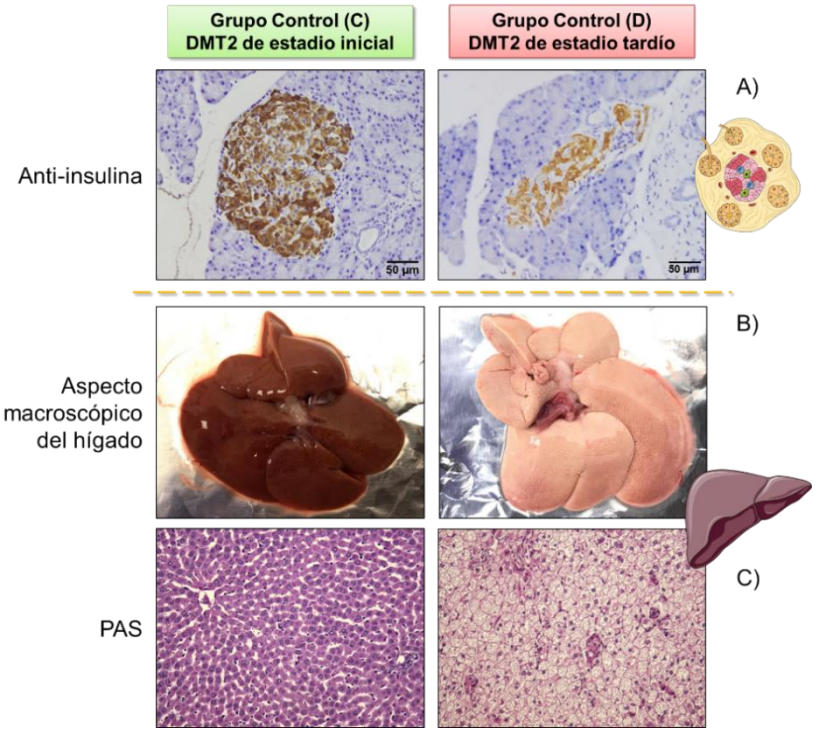

Figura 20. Comparativa entre los grupos control pertenecientes al estadio inicial y tardío de DMT2.

A) Células β pancreáticas por islote de Langerhäns; B) Aspecto mascroscópico del hígado; C) Tinción de PAS del hígado. PAS, Periodic Acid-Schiff.

Como se ha comentado en la sección I, Introducción, el hígado es un órgano clave en la DMT2 al regular la homeostasis energética (Loria y cols., 2013). El estado de la patología también condiciona su posible alteración, siendo causa y consecuencia de las alteraciones sistémicas encontradas. El análisis macroscópico hepático del grupo C no reveló esteatosis en primer término compatible con diagnóstico de NAFLD, aunque sí se observó una leve infiltración microvesicular en el análisis histológico (datos no publicados). En cambio, el estudio histopatológico del hígado del grupo D mostró una gran acumulación de vesículas de lípidos y balonización en los hepatocitos, lo que demuestra las alteraciones asociadas al avanzado estado de la patología y permite el diagnóstico de NASH (Figura 20) (**Artículo 9**) (Dharmalingam & Yamasandhi, 2018). La RI hepática juega un papel esencial en el acúmulo de lípidos, pudiéndose comprobar cómo cuando hay una señalización de insulina todavía eficiente, se favorece la síntesis de glucógeno y se reduce la DNL (grupo C) (**Artículo 7**). En cambio, cuando la sensibilidad a la insulina hepática se ve muy alterada, hay un intercambio en los depósitos del hígado, favoreciéndose el almacenamiento de lípidos frente al de glucógeno (grupo D) (Bechmann y cols., 2012). En este punto también es importante la RI del tejido adiposo que determina el incremento de la lipolisis y la llegada masiva de AGL al hígado, que contibuyen a la aparición de la esteatosis

hepática y el NAFLD. Las marcadas diferencias entre los dos grupos (C y D) se ven influidas en mayor medida por el aporte de colesterol y ácido cólico de la dieta del grupo D, cuyo consumo aceleró la progresión de la patología hepática a NASH (Figura 20). Además, esta diferencia en la composición de la dieta explica tambiénlas alteraciones típicas de la dislipemia diabética encontradas en ambos grupos (**Artículos 7 y 8**). Es bien sabido que el tipo de dieta, la RI, y el desarrollo de NAFLD/NASH condicionan notablemente los cambios cualitativos y cuantitativos de las lipoproteínas (Verges, 2015). Así, las ratas C mostraron hiperlipemia e hipertrigliceridemia, las cuales indujeron un incremento en la producción de $VLDL_1$, fácilmente identificables por el mayor tamaño y enriquecimiento de estas lipoproteínas en triglicéridos (Tomkin & Owens, 2017). En cambio, la inclusión de colesterol en la dieta condicionó notablemente el metabolismo lipoproteico de las ratas D. Estas presentaron elevación de los lípidos plasmáticos, con hipercolesterolemia (colesterol ≥ 100 mg/dL) (Sánchez-Muniz & Bastida, 2008) en el 87,5% de los animales de este grupo, lo que indujo la formación de partículas β-VLDL enriquecidas en colesterol y pobres en triglicéridos (**Artículo 8**) (Cali y cols., 2007; Verges, 2015). No obstante, también se encontraron niveles elevados de la masa total de IDL y LDL, correspondiéndose con estudios previos de nuestro grupo con ratas Wistar alimentadas con dieta hipercolesterolemiante (Garcimartín y cols., 2015b; Olivero-David y cols., 2011; Schultz-Moreira y cols., 2014b).

Tabla 10. Parámetros plasmáticos para definir el estadio de la DMT2.

	C	D	*t* de Student
Glucosa, mg/dL	250,61 ± 16,47	326 ± 29,72	<0,001
Insulina, μIU/mL	15,84 ± 0,73	5,41 ± 1,23	<0,001
HOMA-IR	9,79 ± 0,64	4,57 ± 1,55	<0,001
QUICKI	0,28 ± 0,01	0,31 ± 0,01	<0,001
HOMA-β	30,63 ± 3,40	7,24 ± 1,35	<0,001
Triglicéridos, mg/dL	158,69 ± 21,82	77,63 ± 9,80	<0,001
Colesterol, mg/dL	79,39 ± 10,67	108,18 ± 7,90	<0,001

HOMA-IR, modelo homeostático de resistencia a la insulina; HOMA-β, modelo homeostático de funcionalidad de las células β pancreáticas; QUICKI, índice de control cuantitativo de la sensibilidad a la insulina.

Por otra parte, la evidencia actual señala que la disbiosis intestinal participa activamente en el inicio de la DMT2 (Sikalidis & Maykish, 2020). La dieta es el factor clave que modifica la microbiota, donde una alimentación rica AGS y colesterol modula negativamente las poblaciones bacterianas consideradas beneficiosas, y promueve el

incremento de especies fundamentalmente pertenecientes al filo Firmicutes (**Artículo 6**). Por ello, numerosos autores han señalado el cociente Firmicutes/Bacteroidetes como un indicador de la disbiosis intestinal y del riesgo de DMT2 (Sikalidis & Maykish, 2020). A pesar de que en la presente Tesis Doctoral no se determinó la abundancia de los filos de Firmicutes y Bacteroidetes para poder calcular dicho índice, teniendo en cuenta que los grupos *Blautia coccoides –Eubacterium rectale* y *Clostridium leptum*. representan la mayoría de Firmicutes presentes en las heces, y que *Bacteroides* spp. es el género principal de Bacteroidetes (Schwiertz, 2016), se puede hacer una aproximación a la relación Firmicutes/Bacteroidetes. La comparativa entre ambos modelos animales reveló una menor ratio Firmicutes/Bacteroidetes para el grupo C (2,08 ± 0,9 *vs*. 2,19 ± 0,8, p=0,043), lo que se correlaciona directamente con una menor disbiosis (**Artículo 10**). Estos cambios en la microbiota condicionan la producción de AGCC e inducen permeabilidad intestinal, afectando la estructura y comprometiendo en último término la homeostasis colónica (Sharma & Tripathi, 2019). A falta de ratas control sanas para establecer las alteraciones de ambos grupos, la comparativa entre los grupos C y D, reveló una disfunción de la integridad de barrera, con acortamiento de criptas, menor número de células caliciformes, una capa mucosa reducida, mayor infiltración de células inflamatorias en la lámina propia y un peor estatus antioxidante colónico en las ratas D de estadio avanzado de DMT2 (**Artículos 10 y 11**). Todas estas características son indicativas de una mayor disfunción de la barrera intestinal y un mayor riesgo de endotoxemia metabólica en dicho grupo (**Artículo 6**) (Gurung y cols., 2020; Sircana y cols., 2018; Thaiss y cols., 2018).

Una vez descritas las alteraciones locales de la mucosa colónica, es necesario tener en cuenta que la disbiosis intestinal también promueve cambios a nivel sistémico a través de mediadores como los AGCC o la translocación de LPS (Gomes y cols., 2017; Sikalidis & Maykish, 2020). En relación con este último aspecto, el LPS puede alcanzar el torrente sanguíneo a través de dos vías principales, difusión directa debido al aumento de la permeabilidad paracelular intestinal o por captación e incorporación de LPS a los QM. En la circulación sanguínea se puede unir a los macrófagos e inducir la producción de citoquinas proinflamatorias, capaces de acelerar la disfunción pancreática. A su vez, el LPS también puede alcanzar el hígado y activar cascadas inflamatorias por su unión a TLR4, favoreciendo la progresión de NAFLD a NASH (Gomes y cols., 2017), tal y como se observó en el grupo D (datos no publicados). Por tanto, con esta discusión sobre los modelos animales empleados, se pretende destacar la multifactorialidad de la DMT2 y resaltar la importancia de la nutrición como terapia multidiana.

5.2.2. Impacto del consumo de un cárnico enriquecido en CFE en un modelo murino de estadio inicial de DMT2 (Artículo 7)

La inclusión del cárnico enriquecido en CFE en el modelo murino de estado inicial de DMT2 (grupo CE) indujo una mejora significativa sobre muchas de las alteraciones fisiopatológicas. Las ratas CE revelaron un menor incremento de peso y depósitos grasos, debido principalmente al afecto anorexígeno del CFE (Zhu y cols., 2019). A su vez, se observó un incremento del número de deposiciones y mayor presencia de grasa en heces en comparación con C, lo que corroboró el efecto inhibitorio del CFE sobre la digestión y absorción de grasa previamente descritos en el **artículo 2**. Similares resultados se han apreciado en otros estudios con cárnicos enriquecidos en compuestos bioactivos como silicio o chía, lo que demuestra que el ingrediente funcional es liberado de la matriz cárnica durante el proceso digestivo y exhibe sus diferentes efectos en el tracto gastrointestinal (**Artículo 3**) (Garcimartín y cols., 2015b; Santos-López y cols., 2018). A pesar de que el CFE no indujo cambios significativos en la glucemia final, sí se observó una reducción de la insulina plasmática y HOMA-IR, lo que fue claramente indicativo de una menor RI, tal y como reafirmó el mayor índice QUICKI encontrado en estos animales. De la misma forma, el consumo del cárnico enriquecido en CFE corrigió parcialmente la dislipemia diabética al reducir los lípidos totales, triglicéridos plasmáticos e índice aterogénico, demostrando su potencialidad como agente hipolipemiante y minimizando el riesgo cardiovascular (Ruiz-Roso y cols., 2010; Sánchez-Muniz, 2012). A su vez, el CFE también fue capaz de modular el metabolismo lipoproteico a dos niveles diferentes: a) redujo la concentración de VLDL como consecuencia de una menor disponibilidad de sustratos para ser empaquetados en estas lipoproteínas (triglicéridos y AGL), correlacionado con una menor absorción de lípidos de la dieta (**Artículo 2**); y b) indujo un incremento de la masa total de IDL y LDL, lo que sugiere un mayor aclaramiento a través del LDLr, cuyos niveles fueron significativamente más elevados (**Artículo 7**). Es ampliamente conocido que la insulina es una hormona clave en la regulación de la distribución y metabolismo de los lípidos (Tomkin & Owens, 2017; Verges, 2015). Los efectos anteriormente descritos sobre el metabolismo de las lipoproteínas estuvieron fuertemente influenciados por la menor RI hepática encontrada en las ratas CE. La mejor señalización de insulina justificada por los mayores niveles de la ruta InsR/PI3K/AKT en el hígado, permitieron la acción fisiológica de dicha hormona inhibiendo la fase de maduración y ensamblaje de VLDL y aumentando la expresión y actividad de LDLr (Brown & Gibbons, 2001; Chait y cols., 1979; Sparks y cols., 2012); lo que justifica la mejor homeostasis lipoproteica encontrada en el grupo CE. De forma paralela, el incremento en los niveles de las

proteínas de la vía InsR/PI3K/AKT y GLUT-2 se corresponden con la mejora de los índices HOMA-IR y QUICKI, lo que indudablemente confirma la reducción de la RI tras el consumo del cárnico enriquecido en CFE (**Artículo 7**). Las PACs de otras fuentes vegetales han demostrado su efectividad reduciendo la RI y mejorando el control glucémico (**Artículo 6**) (Cires y cols., 2016; Rauf y cols., 2019; Yang & Chan, 2017; Yogalakshmi y cols., 2014). Respecto a la algarroba, ya hemos comentado que existe evidencia de que algunos extractos de la vaina de esta semilla pueden ejercer un efecto antidiabético a través de sus diferentes componentes, tal y como se ha comentado en la introducción (Goulas y cols., 2016; Zhu y cols., 2019). No obstante, en el extracto estudiado los dos compuestos mayoritarios son la fibra dietética y las PACs, siendo estos polifenoles a los que se les atribuye el mayor efecto sobre la sensibilidad de insulina.

En la figura 21 se detallan los mecanismos por los que el CFE ejerce su efecto antidiabético en este modelo de estado inicial de DMT2.

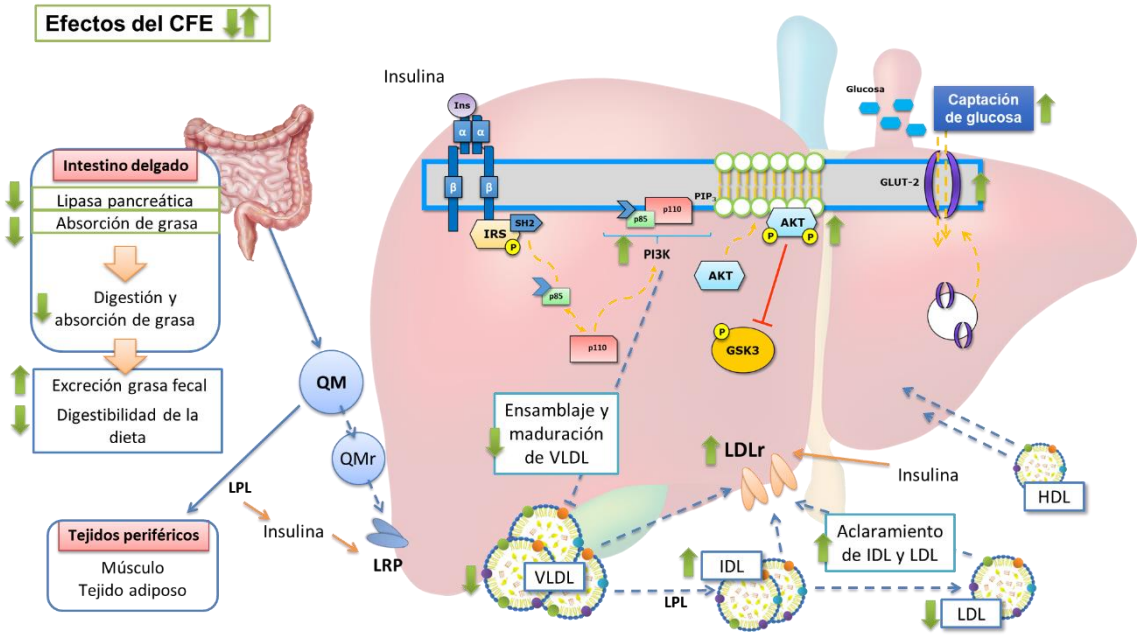

Figura 21. Cambios inducidos por el consumo del cárnico enriquecido en CFE en el grupo CE en comparación con el control C.

CFE, extracto de algarroba (de sus siglas en inglés, *carob fruit extract*); GLUT-2, transportador de glucosa tipo 2; GSK3, glucógeno sintasa quinasa 3; HDL, lipoproteínas de alta densidad; IDL, lipoproteínas de densidad intermedia; Ins, insulina, LDL, lipoproteínas de baja densidad; LPL, lipoprotein lipasa; LDLr, receptor de LDL; PI3K, fosfatidilinositol 3-quinasa; VLDL, lipoproteínas de muy baja densidad. El símbolo ⊥ implica inhibición.

5.2.3. Impacto del consumo de un cárnico enriquecido en CFE en un modelo murino de estadio avanzado de DMT2. ¿Estrategia preventiva o terapéutica? (Artículos 8-11)

La elevada prevalencia de DMT2 y la estimación de la IDF sobre los nuevos casos durante los próximos años a nivel mundial, señalan la importancia de la prevención y el tratamiento de la patología (IDF, 2019). La DMT2 es una enfermedad metabólica caracterizada por un largo periodo de evolución hasta que se confirma el diagnóstico, por ello hay que hacer especial hincapié en las estrategias preventivas que ayuden a ralentizar e incluso detener el progreso (Hays y cols., 2008), entre las que fundamentalmente se encuentran introducir modificaciones del estilo de vida basadas en dieta y ejercicio físico. Ya decía Hipócrates en el siglo V a. C. “Deja tus medicamentos en la olla del químico si puedes curar al paciente con comida”. No obstante, también Hipócrates decía que “El alimento sea tu medicina y que tu medicina sea el alimento” en la idea de que el estatus nutricional puede ser preventivo de enfermedades y reducir o incluso obviar el uso de medicamentos. Por ello uno de los aspectos más relevantes de esta Tesis Doctoral es la comparación de los efectos sobre la fisiopatología de la DMT2 de estadio avanzado del consumo de un cárnico enriquecido en CFE como estrategia preventiva o como estrategia curativa o terapéutica. Los resultados obtenidos revelaron una mejora significativa en muchos de los parámetros estudiados, independientemente de la opción nutricional escogida, remarcando la importancia de incluir en la dieta compuestos naturales como el CFE (Ros y cols., 2015; Xu y cols., 2018).

Para facilitar su comprensión se recuerda que la primera de ellas consistió en administrar el cárnico funcional como estrategia preventiva (grupo ED), es decir, su inclusión en la dieta se realizó desde el inicio del experimento. En cambio, en la segunda estrategia se introdujo el cárnico funcional como tratamiento terapéutico (grupo DE), una vez que se confirmó la hiperglucemia tras la inyección de STZ+NAD.

El consumo del cárnico enriquecido con CFE, tanto de forma preventiva como terapéutica, reveló un potente efecto antidiabético, retrasando la progresión de la DMT2 hacia el estadio tan avanzado observado en el grupo D. La evaluación histológica del páncreas puso de manifiesto el efecto protector del CFE frente al daño inducido por STZ+NAD y la dieta alta en grasas saturadas y colesterol, preservando el número y tamaño de los islotes pancreáticos (Figura 22) (**Artículo 10**). Esta mejora justifica los mayores niveles de insulina e índice HOMA-β encontrados, tal y como han descrito otros autores tras el consumo de extracto de algarroba en estudios en

humanos y en animales (Lambert y cols., 2018). Las PACs de otras fuentes han demostrado ser efectivas frente a la disfunción pancreática presente en la DMT2, reduciendo el estrés oxidativo que conduce al daño celular y la apoptosis (Ding y cols., 2013). Sin embargo, es importante resaltar que los efectos de las PACs sobre el páncreas no se deben mayoritariamente a una acción directa de estos polifenoles, sino que son originados por los metabolitos activos producidos durante la fermentación colónica (**Artículos 6 y 10**).

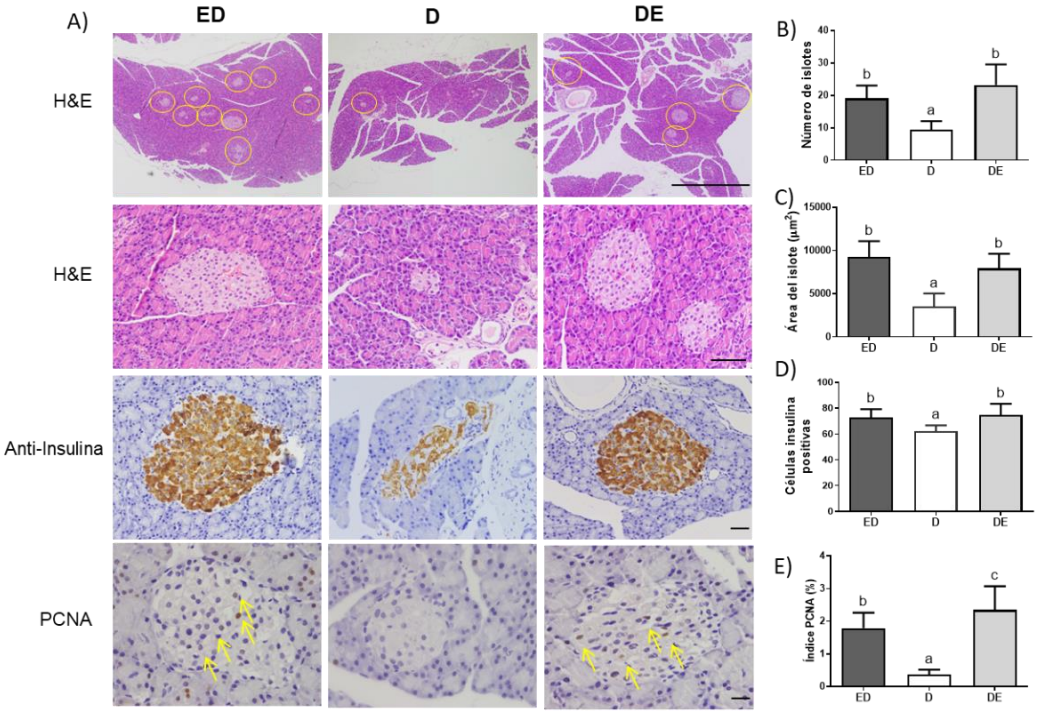

Figura 22. Efecto del consumo crónico del cárnico enriquecido en CFE sobre la disfunción pancreática.

A) Imágenes representativas del páncreas; B) Número total de islotes de Langerhäns por sección pancreática; C) área promedio de los islotes; D) número de células β pancreáticas por islote pancreático, E) índice de proliferación celular. ED, estrategia preventiva; D, control; DE, estrategia terapéutica. Disponible en el artículo 9.

Estos productos secundarios son capaces de realizar modulaciones nutrigenómicas a diferentes niveles, los cuales pueden ser responsables de los mayores niveles de las proteínas participantes en la ruta principal de señalización de insulina (InsR/PI3K/AKT/GSK3) hepática y de la mayor secreción de insulina por parte del páncreas, lo que justificaría la reducción de la glucemia (-13% y -23%) encontrada en las ratas ED y DE, respectivamente, en comparación con el grupo control. Estos resultados van de forma paralela en ambas estrategias nutricionales, permitiendo definir varios mecanismos implicados en la mejor homeostasis de la glucosa tras el consumo de CFE. En primer lugar, el CFE reduce la digestión y absorción de los

hidratos de carbono desde una única administración, lo que es indicativo de una acción directa del CFE (**Artículo 1**). Asimismo, los metabolitos activos generados durante la fermentación son capaces de ejercer modulaciones nutrigenómicas a diferentes niveles, reduciendo la expresión de SGLT1 a nivel intestinal (**Artículo 1**), incrementando la síntesis y secreción de insulina, reduciendo la disfunción pancreática y promoviendo la captación de glucosa hepática a través de una mayor activación de la ruta InsR/PI3K/AKT/GSK3 (**Artículo 7 y 9**) (Cires y cols., 2016; Ding y cols., 2013).

Por otra parte, al evaluar los efectos de ambas estrategias sobre el metabolismo lipídico y lipoproteico, se observó una mejoría más marcada en la estrategia preventiva frente a la terapéutica (**Artículo 8**). Esta afirmación se deduce de: a) que en el el grupo DE un 25% de las ratas eran hipercolesterolémicas (≥100 mg/dL), mientras que en el grupo ED ningún animal alcanzó estas cifras; b) diferencias significativas en la masa global y composición de las partículas VLDL, IDL y LDL. El menor impacto de la dieta alta en grasa saturada y colesterol durante las tres primeras semanas del estudio en el grupo ED, debido a los potentes efectos hipocolesterolemiante e hipolipemiante del CFE (Ruiz-Roso y cols., 2010; Zunft y cols., 2003), explicarían, al menos en parte, las diferencias entre ambos tratamientos (**Artículo 8**). Estos parámetros son un ejemplo claro de la importancia de la prevención, destacando la dificultad de revertir la dislipemia diabética simplemente con un tratamiento nutricional (Jialal & Singh, 2019). En cambio, un mecanismo que se encontró en los dos grupos de tratamiento en comparación con el control, fue la mayor expresión y niveles hepáticos de LDLr. La mayor abundancia de este receptor se ha relacionado de forma inversa con el desarrollo de NAFLD, ya que permite una mayor captación de VLDL, IDL y LDL por parte del hígado, minimizando la permanencia de estas lipoproteínas en sangre y su posibilidad de oxidación (Nakamuta y cols., 2009; Toyota y cols., 1999). Asimismo, cabe recordar que el CFE es un potente antioxidante natural, lo cual quedó demostrado en ambos grupos al incrementar significativamente la actividad AE en plasma e hígado, a la vez que redujo la oxidación hepática (**Artículos 7 y 8**). La AE ha sido definida como una enzima suicida que presenta una actividad antioxidante pleiotrópica, capaz de proteger frente a la oxidación a las diferentes lipoproteínas y tejidos (Nus y cols., 2008; Vázquez-Velasco y cols., 2011). Indudablemente, esta reducción de la oxidación hepática estaría bloqueando, al menos parcialmente, el segundo impacto que se contempla en la progresión de NAFLD a NASH (Day & James, 1998), mejorando a su vez el estado diabético.

El hígado participa activamente en la homeostasis energética, siendo una pieza clave en el desarrollo de DMT2. Su evaluación histológica reveló una menor

acumulación de lípidos y balonización hepática en ambos grupos de tratamiento (ED y DE) en comparación con el grupo D, permitiéndonos situar a este grupo en un estado menos avanzado de NAFLD (**Artículos 8 y 9**, y datos no publicados). Estos resultados son coincidentes con otros previos del grupo AFUSAN en los que se estudiaron diversos ingredientes funcionales como silicio, chía o glucomanano (Garcimartín y cols., 2015b; Santos-López y cols., 2017; Vázquez-Velasco y cols., 2017). Se han evaluado diferentes mecanismos que podrían estar implicados en este efecto hepatoprotector, los cuales se describen en relación con el grupo control D. En primer lugar, tanto el efecto inhibitorio del CFE sobre la digestión y absorción de grasas (**Artículo 2**), como su marcada actividad hipotrigliceridemiante e hipocolesterolemiante (**Artículo 8**), reduciría la entrada de sustratos lipídicos al hígado. El segundo aspecto a destacar es la mejora de la señalización de insulina hepática, promoviendo la formación de glucógeno a través de la interacción de GSK3 y GS. En tercer lugar se encuentra la reducción de la DNL tras el consumo del cárnico enriquecido en CFE, produciendo una disminución de los factores de transcripción lipogénicos LXRα/β, SREBP-1c y ChREBP (**Artículo 9**). El cuarto aspecto relevante se relaciona con los mayores niveles de insulina observados tanto en los animales del grupo preventivo como los del terapéutico, pudiendo ejercer una mayor acción inhibitoria sobre la lipasa sensible a hormonas (LSH), reduciendo la lipolisis del tejido adiposo y frenando la salida de AGL hacia el hígado (datos en estudio). Por último, la mayor eliminación de triglicéridos en VLDL, también puede ser responsable de estos efectos protectores frente al desarrollo de NAFLD. Sin embargo, este último mecanismo solo lo podemos asociar con el grupo ED, debido a que en el grupo DE no se observaron grandes cambios (**Artículos 7 y 8**). Sería adecuado evaluar el efecto del CFE sobre la β-oxidación y los niveles de AGL circulantes para poder responder con certeza a estas diferencias (Bechmann y cols., 2012; López-Oliva & Muñoz Martínez, 2014).

La fisiopatología de la DMT2 también se asocia con alteraciones de la microbiota intestinal y la permeabilidad intestinal (**Artículos 6, 10 y 11**). Los resultados relativos a estos puntos revelaron marcadas diferencias en la integridad de la barrera colónica, incluyendo a la microbiota como componente clave en la permeabilidad intestinal (**Artículo 6**). La comparativa entre el grupo preventivo y terapéutico ha permitido observar y resaltar que el efecto prebiótico atribuido de forma generalizada a la fibra y las PACs parece depender de la situación fisiopatológica y tiene que ser evaluado en cada uno de los estudios. Así, las ratas del grupo preventivo (ED) presentaron mayor abundancia y diversidad de especies bacterianas beneficiosas (*Bacteroides* spp., *Bifidobacterium* spp., F. *prausnitzii* y *Lactobacillus* spp.) en detrimento de aquellos

potencialmente perjudiciales (*Enterobacteriaceae*) (**Artículo 10**) (Cani y cols., 2008; Hildebrandt y cols., 2009) respecto al control D; mientras que el consumo terapéutico no indujo grandes modificaciones (**Artículo 10**). Curiosamente, solo se observó paralelismo entre ED y DE, con una reducción similar de los niveles de del grupo *Blautia coccoides-Eubacterium rectale*, el cual se ha asociado de forma negativa con el grado de desarrollo de DMT2 y el control glucémico (Egshatyan y cols., 2016). Estos cambios se corresponden con la mejora metabólica encontrada en estos animales en comparación con el grupo control (**Artículo 9**). Todos estos datos sugieren que el CFE al mismo tiempo que ejerce un efecto prebiótico, puede minimizar la disbiosis asociada a la DMT2, fundamentalmente bloqueando el impacto de la dieta alta en grasas saturadas y colesterol. No obstante, una vez que está instaurada la patología y se presentan alteraciones de la microbiota, es más complicado ejercer efectos beneficiosos con las pequeñas dosis de CFE incluidas o se requeriría un tratamiento más prolongado. Esta afirmación queda reforzada al compararse la morfología del colon entre los grupos alimentados con el cárnico enriquecido en CFE. Así, el grupo de prevención mostró una mayor capa mucosa, profundidad de las criptas y número de células caliciformes en comparación con el grupo terapéutico y el control (Figura 23) (**Artículo 10**), lo que apoya el binomio microbiota-integridad de la barrera colónica (**Artículo 6**).

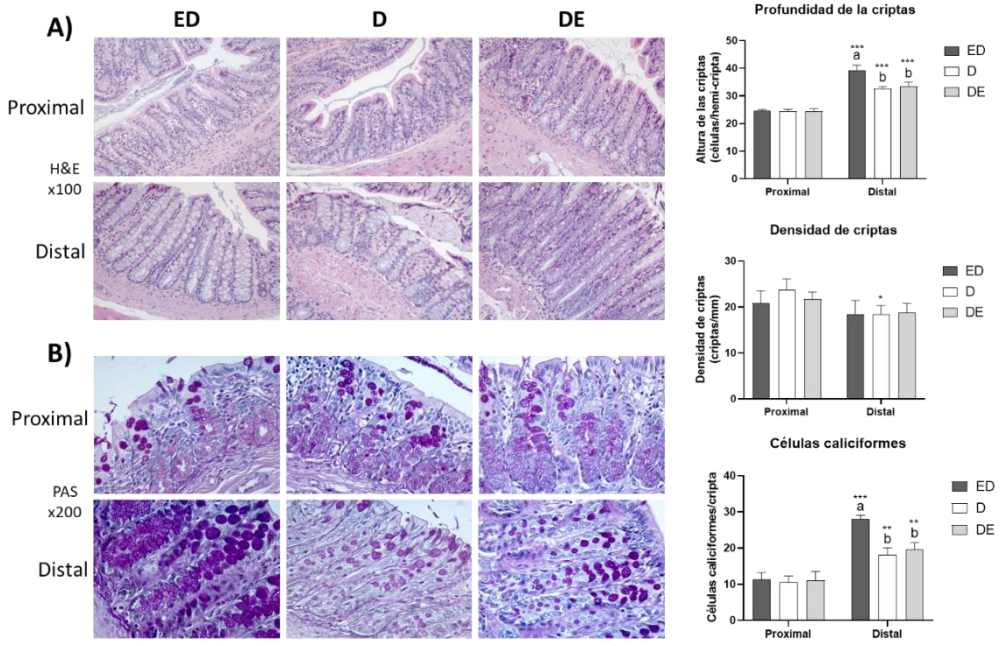

Figura 23. Efecto del consumo del cárnico enriquecido en CFE sobre la morfología colónica.

A) Tinción de H&E (evaluación de la profundidad y densidad de criptas); B) Tinción de PAS (Periodic Acid-Schiff) del colon (cuantificación de las células caliciformes). ED, estrategia preventiva; D, control; DE, estrategia terapéutica. Disponible en el artículo 10.

Por otra parte, las uniones estrechas también son un pilar esencial de la barrera colónica. Así, es importante destacar el incremento del 72,6% y 177,1% de la expresión de ocludina y la reducción del 51,1% y 30% de los niveles de zonulina-1 del colon distal en el grupo ED y DE, respectivamente, en comparación con las ratas D. El incremento exacerbado de zonulina se ha asociado con la disbiosis intestinal derivada de dieta con alto contenido en grasa, llegándose a proponer como un marcador en sangre predictivo de la diabetes por su gran correlación con la permeabilidad intestinal (Mokkala y cols., 2017; Palacios y cols., 2020; Sturgeon & Fasano, 2016). Estos resultados sugieren un refuerzo de las uniones estrechas, posiblemente en un intento de revertir la permeabilidad colónica y frenar los efectos deletéreos de la dieta alta en grasas y colesterol. Unido a ello, la defensa antioxidante del colonocito se ha propuesto como un mecanismo esencial en la barrera colónica (Rohr y cols., 2020) (**Artículo 6**), ya que es necesaria para contrarrestar la continua llegada de compuestos oxidados procedentes de la dieta (**Artículos 4 y 6**). Así, el consumo preventivo y terapéutico del cárnico enriquecido en CFE indujo una mayor expresión y actividad de la maquinaria antioxidante en la mucosa colónica proximal en relación con el grupo control D. Este incremento se debió fundamentalmente a una mayor translocación del factor de transcripción Nrf2 al núcleo (**Artículo 11**). De forma constitutiva, dicho factor se encuentra inactivo en el citoplasma, unido a una Keap1, proteína represora que contribuye a la degradación del complejo Nrf2-Keap1 por el proteasoma. En cambio, su fosforilación en serina40 permite su liberación y translocación al núcleo, regulando la expresión génica a través de interacciones con el elemento de respuesta antioxidante, que se encuentra en las regiones promotoras de muchos genes de defensa celular (Long y cols., 2016; Yang y cols., 2018). Las PACs y sus metabolitos activos han demostrado ser eficaces modulando la maquinaria antioxidante a través de esta ruta de señalización (Li y cols., 2015).

Finalmente, con el objetivo de identificar mejor el estatus colónico entre grupos, se realizó un score o puntuación agrupando los parámetros más importantes (microbiota, morfología colónica, AGCC y proteínas de unión estrecha) (**Artículo 10**). La agrupación de variables permite una mejor separación de los dos tratamientos y categorizar mejor la patología que un marcador aislado (Gesteiro y cols., 2013; Ruperto y cols., 2017). Como era de esperar, el grupo ED mostró la mejor puntuación; situándose, el grupo DE en un punto intermedio entre el grupo ED y el control D, lo que es indicativo de una corrección parcial de las alteraciones de la integridad de la barrera colónica. Por ello, de estos resultados se puede deducir que el CFE: a) bloquea la disbiosis inducida por la dieta alta en grasas al ejercer un efecto prebiótico,

lo que ayuda a mantener niveles adecuados de especies bacterianas beneficiosas y AGCC; y b) ejerce un efecto directo sobre la mucosa colónica, reforzando las uniones estrechas, promoviendo el recambio celular e incrementando el sistema antioxidante. Por tanto, nuestros datos señalan la importancia de consumir una dieta adecuada para prevenir el desarrollo de la disbiosis intestinal y sus complicaciones asociadas (Gurung y cols., 2020; S. Sharma & Tripathi, 2019); siendo necesario evitar generalizaciones y considerar el estado de la patología en cada uno de los estudios para poder obtener conclusiones relevantes.

Finalmente, en la figura 24 se resumen los efectos del consumo del cárnico con CFE en este modelo de estadio tardío de DMT2, tanto la estrategia preventiva (ED) como la terapéutica (DE), en ambos casos comparados con el control (D). Utilizando el mismo esquema se puede comprar que las principales diferencias entre una estrategia y otra se centran en el metabolismo lipoproteico e integridad de la barrera intestinal.

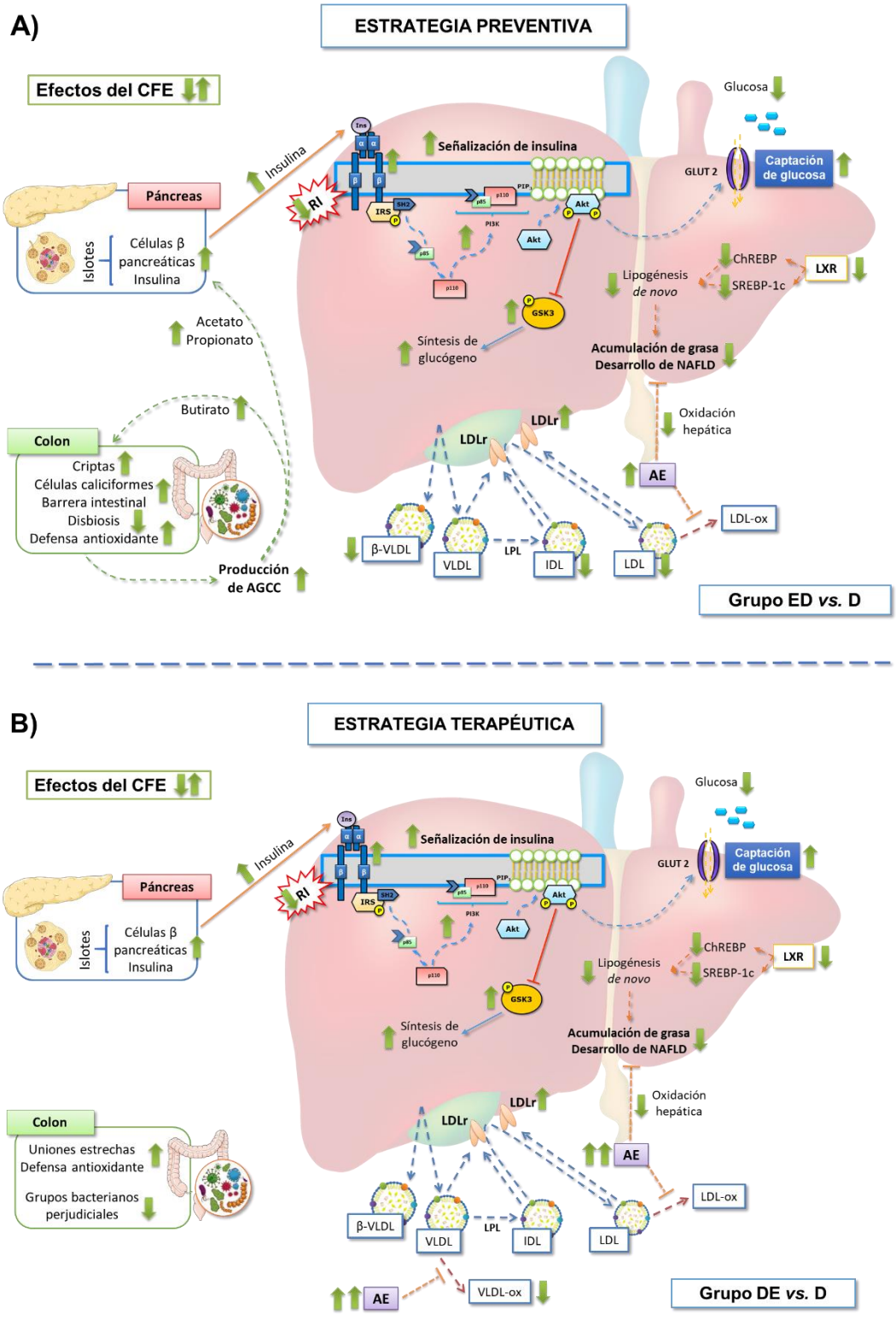

Figura 24. Cambios inducidos por el consumo del cárnico enriquecido en CFE en comparación con el control D.

A) Comparativa del grupo preventivo ED *vs*. control D; B) Comparativa del grupo terapéutico DE *vs*. control D; AE, arilesterasa; AGCC, ácidos grasos de cadena corta; CFE, extracto de algarroba (de sus siglas en inglés, carob fruit extract); ChREBP, proteína de unión al elemento de respuesta a carbohidratos; GLUT-2, transportador de glucosa tipo 2; GSK3,

glucógeno sintasa quinasa 3; HDL, lipoproteínas de alta densidad; IDL, lipoproteínas de densidad intermedia; Ins, insulina, LDL, lipoproteínas de baja densidad; LDLr, receptor para LDL; LDL-ox, LDL oxidadas; LXR, receptor X hepático; LPL, lipoprotein lipasa; PI3K, fosfatidilinositol 3-quinasa; RI, resistencia a la insulina; SREBP-1c, proteína de unión al elemento regulador del esterol; β-VLDL, lipoproteínas de muy baja densidad enriquecidas en colesterol; VLDL, lipoproteínas de muy baja densidad. El símbolo ⊥ implica inhibición.

VI. RESUMEN Y CONCLUSIONES

La Diabetes Mellitus tipo 2 (DMT2) es una patología crónica en la que la modificación de los hábitos de vida, particularmente los relacionados con la dieta, siguen siendo el pilar fundamental del tratamiento. El consumo de compuestos bioactivos de origen natural ha demostrado ser una estrategia nutricional efectiva para conseguir un mejor control glucémico y lipémico, lo que minimiza el impacto de la DMT2 y sus complicaciones en la salud

El algarrobo (*Ceratonia siliqua*, L.) es un árbol de la familia de las fabáceas muy extendido en la zona Mediterránea, cuyo fruto ha demostrado tener numerosas aplicaciones tecnológicas y nutricionales. De hecho, existe evidencia científica que relaciona su consumo, debido a su alto contenido en fibra y proantocianidinas (PACs), con una mejora de las enfermedades crónico-degenerativas más prevalentes en los países desarrollados, como es el caso de DMT2. Estas mejoras se deben fundamentalmente a sus efectos antioxidantes, anticancerígenos, hipolipemiantes y antidiabéticos.

Esta Memoria de Tesis Doctoral se ha desarrollado con la finalidad de conocer los efectos del consumo de un extracto de algarroba (CFE, de sus siglas en inglés *Carob Fruit Extract*) como complemento alimenticio o formando parte de un cárnico funcional en diferentes modelos de rata e identificar y caracterizar los mecanismos moleculares implicados. Para ello, se han realizado tres experimentos en ratas Wistar. El primero de ellos en animales jóvenes y sanos para verificar los efectos del CFE sobre la digestión y absorción de hidratos de carbono y grasas; y posteriormente en dos modelos de DMT2, que corresponden a los estadios inicial y final de la patología, en los que se evalúan los efectos del consumo crónico de un cárnico funcional enriquecido en CFE.

Los resultados obtenidos han permitido establecer las siguientes **conclusiones**:

1. El CFE puede considerarse un complemento alimenticio, por ser fuente de fibra y PACs. El análisis mediante resonancia magnética nuclear señala la presencia mayoritaria de prodelfinidinas B3 y C2 conformando estructuras poliméricas de 3 a 9 unidades.

2. Los estudios *in vitro* señalan que el CFE inhibe de forma dosis-dependiente la actividad de la α-glucosidasa (actividad maltasa) y de la lipasa pancreática, enzimas clave en la digestión de los hidratos de carbono y las grasas.
3. La administración aguda y subcrónica de CFE a ratas jóvenes sanas reduce de forma dosis-dependiente las áreas bajo la curva postprandiales de glucemia y de

lipemia, como consecuencia a) de la reducción de la digestión de hidratos de carbono y grasas, b) del efecto arrastre de la fibra insoluble incrementando excreción de macronutrientes en heces; y c) de la disminución de la expresión génica de transportadores clave como el SGLT1, responsable de la absorción de glucosa.

4. Cuando se incluye el CFE en sistemas basados en emulsiones simples o gelificadas formuladas con aislado de proteína de suero de la leche, básicas en el desarrollo de cárnicos funcionales, este ingrediente funcional mantiene sus propiedades antioxidantes e inhibitorias de la lipasa pancreática.
5. El empleo del CFE como ingrediente funcional incluido en una matriz cárnica es una estrategia tecnológica adecuada que mantiene el valor nutricional del producto cárnico, permite incrementar el aporte de fibra y PACs, y contrarrestar los efectos prooxidantes atribuidos a la carne.
6. El consumo de una dieta rica en grasas saturadas incluyendo un cárnico control induce hiperglucemia e hiperinsulinemia, compatibles con un estadio inicial de DMT2; mientras que la inclusión de colesterol y ácido cólico en esta dieta en combinación con una inyección de estreptozotocina y nicotinamida induce hiperglucemia e hipoinsulinemia, debida a la destrucción de células β pancreáticas, compatibles con un estadio avanzado de DMT2.
7. El consumo de un cárnico conteniendo CFE en el estadio inicial de la DMT2 como estrategia preventiva mantiene la sensibilidad a la insulina en el hígado a través de la vía de señalización InsR/IRS/PI3K/AKT, lo que se traduce en una menor hiperinsulinemia y dislipemia diabética.
8. El tipo de estrategia nutricional empleada, preventiva o terapéutica, determina la eficacia del cárnico con CFE añadido sobre la fisiopatología de la DMT2 avanzada.
9. En el estadio avanzado de DMT2, el consumo preventivo del cárnico conteniendo CFE evitó la progresión de la patología, mostrando una mayor funcionalidad pancreática y sensibilidad a la insulina en el hígado, menor dislipemia y esteatosis hepática; y menor disbiosis, estrés oxidativo y permeabilidad colónica.
10. En el estadio avanzado de DMT2, el consumo terapéutico del cárnico con CFE revirtió y ralentizó la progresión de la patología al mantener una mayor sensibilidad hepática a la insulina, menor disfunción pancreática y esteatosis hepática, y mejor estatus antioxidante colónico.

Conclusión general y futuras aplicaciones

El consumo elevado de carne y productos cárnicos se ha asociado con el incremento de enfermedades crónicas muy prevalentes. Los resultados de esta Tesis Doctoral justifican la inclusión de CFE a una matriz cárnica con el objetivo de elaborar un cárnico con propiedades antidiabéticas y capacidad para mitigar los aspectos negativos asociados al consumo de cárnicos comerciales, especialmente en lo relativo a su carácter prooxidante. El consumo del cárnico enriquecido en CFE ejerce un efecto antidiabético multidiana, reduciendo la glucemia y la lipemia, protegiendo la funcionalidad del páncreas y el hígado, mejorando el estatus antioxidante, la disbiosis y la integridad de la barrera colónica; aspectos que sugieren ampliamente que se trata de un alimento que mejora funcionalidades y minimiza el riesgo de desarrollar DMT2. Dada la relevancia de los resultados encontrados en los modelos experimentales de DMT2, preferentemente cuando se consume el cárnico enriquecido en CFE como estrategia preventiva, parece importante desarrollar nuevos estudios donde se compruebe la reproducibilidad de los resultados, tanto en mujeres como en hombres, con diferente grado de evolución de la patología para poder elevar declaraciones de propiedades saludables.

VII. SUMMARY AND CONCLUSIONS

Type 2 Diabetes Mellitus (T2DM) is a chronic pathology in which lifestyle habit modification, particularly those related to diet, continue to be the fundamental pillar of treatment. The consumption of natural origin bioactive compounds has proven to be an effective nutritional strategy to achieve better glycemic and lipemic control, which minimizes the impact of T2DM and its health complications.

The carob tree (*Ceratonia siliqua*, L.) is a tree in the Fabaceae family that is widespread in the Mediterranean area, whose fruit has shown numerous technological and nutritional applications. In fact, there is scientific evidence that links its consumption, due to its high content of fiber and proanthocyanidins (PACs), to an improvement in the most prevalent chronic-degenerative diseases in developed countries, such as T2DM. These improvements are mainly due to its antioxidant, anticancer, lipid-lowering, and antidiabetic effects.

This Doctoral Thesis has been developed with the purpose of understanding the effects of consuming a Carob Fruit Extract (CFE) as a food supplement or as a functional meat ingredient in different rat models, and identify and characterize the molecular mechanisms involved. In order to accomplish this, three experiments have been carried out on Wistar rats. The first of them was carried out in young and healthy animals to verify the CFE effects on the digestion and absorption of carbohydrates and fats; the other two were performed in two T2DM models, corresponding to the initial and final stages of the pathology, in which the effects of chronic CFE-enriched meat consumption were evaluated.

The results obtained establish the following **conclusions**:

1. CFE can be considered a dietary supplement, as it is a source of fiber and PACs. Nuclear magnetic resonance analysis indicates the majority presence of prodelphinidins B3 and C2, forming polymeric structures of 3 to 9 units.
2. *In vitro* studies indicate that CFE dose-dependently inhibits the activity of α-glucosidase (maltase activity) and pancreatic lipase, key enzymes in carbohydrate and fat digestion.
3. The acute and subchronic CFE administration to healthy young rats dose-dependently reduces the areas under the curve of postprandial glycemia and lipemia, as a consequence of a) the reduction of carbohydrate and fat digestion; b) the entrainment effect of insoluble fiber increasing macronutrient fecal excretion; and c) the decrease in gene expression of key transporters such as SGLT1, responsible for glucose uptake.

4. When CFE is included in systems based on simple or gelled emulsions formulated with whey protein isolate, basic in the development of functional meats, this ingredient maintains its antioxidant and inhibitory properties of pancreatic lipase.
5. The use of CFE as a functional ingredient included in a meat matrix is an appropriate technological strategy that maintains the value of the nutritional meat, allows for an increase in the contribution of fiber and PACs, and counteracts the pro-oxidant effects attributed to the meat.
6. The consumption of a diet rich in saturated-fat, including a control meat, induces hyperglycemia and hyperinsulinemia, compatible with an early-stage of T2DM. Conversely, the inclusion of cholesterol and cholic acid in this diet, in combination with a streptozotocin and nicotinamide injection, induces hyperglycemia and hypoinsulinemia, due to the destruction of pancreatic β cells, similar to late-stage T2DM.
7. The consumption of CFE-enriched meat in early-stage T2DM as a preventive strategy maintains insulin sensitivity in the liver through the InsR/IRS/PI3K/AKT signaling pathway, which translates into lower hyperinsulinemia and diabetic dyslipidemia.
8. The type of nutritional strategy used, preventive or therapeutic, determines the efficacy of CFE-enriched meat on late-stage T2DM pathophysiology.
9. In late-stage T2DM, the preventive consumption of CFE-enriched meat blocked the progression of the pathology, showing greater pancreatic functionality and insulin sensitivity in the liver, less dyslipidemia, and hepatic steatosis, as well as less dysbiosis, oxidative stress, and colonic permeability.
10. In late-stage T2DM, the therapeutic consumption of CFE-enriched meat reversed and slowed down the progression of the pathology by maintaining greater hepatic sensitivity to insulin, less pancreatic dysfunction and hepatic steatosis, and better colonic antioxidant status.

General conclusion and future applications

A high consumption of meat and meat products has been associated with an increase in highly prevalent chronic diseases. The results of this Doctoral Thesis justify the inclusion of CFE in a meat matrix in order to elaborate meat with antidiabetic properties along with the capacity to mitigate the negative aspects associated with commercial meat consumption, especially regarding its pro-oxidant character. The

consumption of CFE-enriched meat exerts a multi-target antidiabetic effect, reducing glycemia and lipemia, protecting the functionality of the pancreas and liver, and improving the antioxidant status, dysbiosis, and the integrity of the colonic barrier. Such aspects widely suggest that CFE-enriched meats improve functionalities and minimize the risk of developing T2DM. Given the relevance of the results found in the T2DM experimental models, preferably when the CFE-enriched meat is consumed as a preventive strategy, it proves important to develop new studies in which the reproducibility of the results is verified, both in women and in men, with different degrees of pathological evolutions to be able to claim specific health properties.

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